"EXPRESSION OF SURVIVIN IN ORAL POTENTIALLY MALIGNANT DISORDERS - A RETROSPECTIVE IMMUNOHISTOCHEMICAL STUDY"

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In partial fulfillment of the requirements

For the degree of

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BRANCH VI

ORAL PATHOLOGY & MICROBIOLOGY

THE TAMILNADU DR.M.G.R. MEDICAL UNIVERSITY

CHENNAI – 600032

2015 - 2018

DECLARATION BY THE CANDIDIATE

I hereby declare that this dissertation titled "**EXPRESSION OF SURVIVIN IN ORAL POTENTIALLY MALIGNANT DISORDERS - A RETROSPECTIVE IMMUNOHISTOCHEMICAL STUDY**" is a bonafide and genuine research work carried out by me under the guidance of **DR.C.R.MURALI, M.D.S., Professor, Head of the Department,** Department of Oral Pathology & Microbiology, Best Dental Science College, Madurai - 625104.

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CERTIFICATE BY THE GUIDE

This is to certify that **DR.M.RAJANNA VENKATRAMAN,** Post Graduate student (2015 - 2018) in Department of Oral Pathology and Microbiology, Best Dental Science College, Madurai has done this dissertation titled **"EXPRESSION OF SURVIVIN IN ORAL POTENTIALLY MALIGNANT DISORDERS – A RETROSPECTIVE IMMUNOHISTOCHEMICAL STUDY"** under my direct guidance and supervision in partial fulfillment of the regulations laid down by the Tamilnadu DR.M.G.R. Medical University Chennai – 600032, in Oral pathology and Microbiology (Branch VI) for MDS Degree Examination.

23/01/18

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Modern medical advances have helped millions of people live longer, healthier lives. We owe these improvements to decades of investment in medical research

– Ike Skelton

Without the support, patience and guidance of the following people, this study could not have been completed. It is to them that I owe my deepest gratitude.

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mother for their able support, and to **A.Anushya,** my beloved wife, and **Aadhan Nilavan,** my son, who provided me with unfailing support and endless love.

CERTIFICATE - II

This is to certify that this dissertation work titled **Expression of Survivin in oral potentially malignant disorders – a retrospective immunohistochemical study** of the candidate **Dr.M.Rajanna Venkatraman** with registration number **241521352** for the award of **Master of Dental Surgery** in the branch of **Oral Pathology and Microbiology - Branch VI**. I personally verified the urkund.com website for the purpose of plagiarism check. I found that the uploaded thesis file contains from introduction to conclusion pages and result shows **2** percentage of plagiarism in the dissertation.

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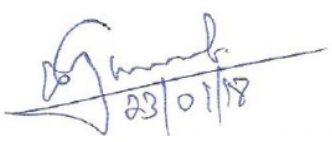
DECLARATION

TITLE OF DISSERTATION	**EXPRESSION OF SURVIVIN IN ORAL POTENTIALLY MALIGNANT DISORDERS – A RETROSPECTIVE IMMUNOHISTOCHEMICAL STUDY**
PLACE OF STUDY	**BEST DENTAL SCIENCE COLLEGE, MADURAI – 625104**
DURATION OF THE COURSE	**3 YEARS**
NAME OF THE GUIDE	**DR.C.R.MURALI MDS**
HEAD OF THE DEPARTMENT	**DR.C.R.MURALI MDS**

I hereby declare that no part of the dissertation will be utilized for gaining financial assistance/any promotion without obtaining prior permission of the Principal, Best Dental Science College, Madurai – 625104. In addition, I declare that no part of this work will be published either in print or in electronic media without the guide who has been actively involved in dissertation. The author has the right to reserve for publish of work solely with the prior permission of the Principal, Best Dental Science College, Madurai – 625104.



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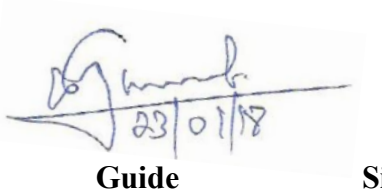

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This agreement herein after the "Agreement" is entered into on this day of 23/1/2018 between the Best Dental Science College represented by its Principal having address at Best Dental Science College, Madurai – 625104. (Hereafter referred to as "the college").

And

DR.C.R.MURALI M.D.S., aged 45 years, working as Professor and Head of the Department at the college, having residence address at CT – 2, Arun C block, Vindhyachal Apartment, Chanda Gandhi Nagar, Bypass Road, Madurai – 625010. Herein after referred to as the Principal Investigator.

And

DR.M.RAJANNA VENKATRAMAN, aged 36 years, studying as postgraduate student in the Department of Oral Pathology and Microbiology in Best dental Science College. Herein after referred to as the PG/research student and co-investigator.

Whereas PG / Research student as part of his curriculum undertakes to research **"Expression of survivin in oral potentially malignant disorders – a retrospective immunohistochemical study"** for which purpose PG/Principal Investigator shall act as Principal Investigator and the college shall provide requisite infrastructure based on availability and also provide facility to the PG /Research student as to the extent possible as a Co–investigator.

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PG student

Witnesses

1. N.V. Vani

Dr. N.V.VANI MDS.,
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2. Dr. A. VASAKI, MDS, SENIOR LECTURER

Student guide

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LIST OF ABBREVIATIONS

BAX	Bcl Associated X
bcl-2	B Cell Lymphoma 2
BCL-XL	BCL Extra large
BIR	Baculovirus IAP Repeat
CIS	Carcinoma in situ
DAB	3,3'-Diaminobenzidine
DNA	Deoxyribo Nucleic Acid
DPX	Distyrene Plasticizer Xylene
ELISA	Enzyme Linked Immuno Sorbent Assay
FFPE	Formalin Fixed Paraffin Embedded
g	Gram
G_0	Gap_0
G_1	Gap_1
G_2	Gap_2
H_2O_2	Hydrogen Peroxide
HCl	Hydrochloric acid
HeLa cells	Henrietta Lacks Cells
HNSCC	Head and Neck Squamous Cell Carcinoma
HPV	Human Papilloma Virus
IAP	Inhibitor of Apoptosis
IHC	Immunohistochemistry

IRS	Immunoreactive score
L	Litre
LP	Lichen Planus
M	Mitosis
MDSCC	Moderately Differentiated Squamous Cell Carcinoma
Min	Minute
mL	Milliliter
MPF	M-phase Promoting Factor
mRNA	Messenger Ribonucleic Acid
N	Normality
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
NES	Nuclear Export Signal
NOE	Normal Oral Epithelium
NOM	Normal Oral Mucosa
OED	Oral Epithelial Dysplasia
OL	Oral Leukoplakia
OLP	Oral Lichen Planus

OPMD	Oral Potentially Malignant Disorders
OSCC	Oral Squamous Cell Carcinoma
OSMF	Oral Submucous Fibrosis
pAkt	Phosphorylated protein kinase strain AK Thymoma
pH	Potential of Hydrogen
PCR	Polymerase Chain Reaction
PDSCC	Poorly Differentiated Squamous Cell Carcinoma
RNA	Ribonucleic Acid
SCC	Squamous Cell Carcinoma
TBS	Tris Buffer Saline
WDSCC	Well Differentiated Squamous Cell Carcinoma
WHO	World Health Organization

LIST OF TABLES

LIST OF GRAPHS

LIST OF FIGURES

TABLE OF CONTENTS

Introduction

INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer in the world. More deaths have been documented due to HNSCC in comparison to cervical cancer, melanoma, or Hodgkin's lymphoma. The 5-year survival rate is only 50% which is poor in comparison to breast cancer or melanoma.[1]

Oral cancer is a subgroup of head and neck malignant neoplasms that includes carcinomas arising from the mucosal lining of the lips, the buccal mucosa, the retromolar trigone, the alveolar ridges, the anterior two- thirds of the tongue, the floor of the mouth, and the hard palate. Although oropharynx refers to the posterior third of the tongue, the soft palate and uvula, the tonsils, and the upper part of the posterior pharyngeal wall, malignant neoplasms involving oral cavity and oropharynx have been categorized together in the codes C00-C14 in the World Health Organization (WHO) International Classification of Diseases, 10th Revision. Numerous types of malignant neoplasms affect the oral cavity and oropharynx, but squamous cell carcinoma (SCC) that arises from the mucosal epithelial lining, accounts for more than 90% of cases.[1]

In 2012, there were 369,200 new cases of oral cancer reported worldwide, with two-thirds of them being diagnosed in developing countries. These tumors were responsible for approximately 145,328 deaths worldwide per year. The areas characterized by high incidence rates for oral cancer (excluding lip) are found in the South and Southeast Asia (e.g. Sri Lanka, India, Pakistan, and Taiwan), parts of Western (e.g. France) and Eastern Europe (e.g. Hungary, Slovakia, and Slovenia), parts of Latin America and the Caribbean (e.g. Brazil, Uruguay, and Puerto Rico) and in Pacific regions (e.g. Papua New Guinea and Melanesia).[1]

INTRODUCTION

Oral cancer is the most common cancer in men in high-risk countries such as Sri Lanka, India, Pakistan, and Bangladesh, and contributes up to 25% of all new cases of cancer. The 5 year survival rate for patients with early localized disease is 80% and that with distant metastases is 19%. The aetiology of oral cancer is multifactorial. The most important risk factors being tobacco, excess consumption of alcohol, and betel quid usage, factors which act separately and synergistically.[2]

Oral epithelial dysplasia (OED) is a common precursor of oral cancer. The occurrence of oral squamous cell carcinoma (OSCC) is preceded by visible changes of the oral mucosa. Longitudinal studies of rural populations in India have revealed that 80% of oral cancers are preceded by precancerous conditions or lesions.[3]

The WHO has recently recommended abandoning the distinction between potentially malignant lesions and potentially malignant conditions and has coined the term potentially malignant disorders. The term 'oral potentially malignant disorders' (OPMD) was adopted by the WHO in 2005 to describe oral lesions and conditions associated with a risk of malignant transformation.[4]

In the WHO collaborating workshop conducted in 2007, the disorders of concern were leukoplakia, erythroplakia, oral submucous fibrosis, oral lichen planus, palatal lesion of reverse smoking of tobacco, discoid lupus erythematosus, and hereditary disorders like dyskeratosis congenital and epidermolysis bullosa.[5]

Oral leukoplakia (OL) is defined as a predominantly white lesion of the oral mucosa that cannot be characterized as any other definable lesion.[6] In 2007, the WHO rephrased the definition as white plaques of questionable risk, (other) known diseases and disorders that carry no increased risk of cancers having been excluded.[4]

The WHO has established criteria for dysplasia, including the architectural and cytologic changes in the epithelium. The WHO's criteria for architectural changes in the epithelium includes irregular epithelial stratification, loss of polarity of basal cells, drop-shaped rete ridges, increased number of mitotic figures, abnormal mitoses not limited to basal or parabasal layers, premature keratinization in single cells (dyskeratosis), keratin pearls within rete ridges. The criteria for cytologic changes in the epithelium include abnormal variation in nuclear size (anisonucleosis), abnormal variation in nuclear shape (nuclear pleomorphism), abnormal variation in cell size (anisocytosis), abnormal variation in cell shape (cellular pleomorphism), increased nuclear–cytoplasmic ratio, increased nuclear size, atypical mitotic figures, increased number and size of nucleoli, and hyperchromasia.[7]

Grading dysplasia depends on the extent of involvement of the epithelial layers by the dysplastic changes. In cases of mild dysplasia, cytologic and architectural changes are confined to the lower third of the thickness of the epithelium; in cases of moderate dysplasia, changes are seen in up to two-thirds of the thickness of the epithelium. In cases of severe dysplasia, the dysplastic changes fill more than two-thirds of the thickness, but less than the entire thickness of the epithelium. The dysplastic cells of carcinoma-in-situ (CIS) occupy the entire thickness of the epithelium (bottom to top changes), although the basement membrane is still intact.[8]

Lichen planus (LP) is defined as an inflammatory disease of chronic nature, affecting skin and mucous membranes. The prevalence of LP is estimated at 0.22% to 5% worldwide, and the incidence of oral lichen planus (OLP) is estimated at up to 2.2%. Oral lesions are seen in around 60% of patients with cutaneous LP. However, in

patients with predominant OLP, cutaneous lesions develop in only 15% of the patients.

Microscopic features of OLP include hyperparakeratosis, hyperorthokeratosis, and combinations of the two; cytoid (Civatte) bodies; basal cell hydropic change; and a band-like chiefly lymphocytic infiltrate in the lamina propria. Additional findings include saw-tooth rete ridges, atrophy, acanthosis, a homogeneous eosinophilic deposit at the epithelium-connective tissue junction, and ulceration. Compared to cutaneous disease, oral lesions less often exhibit saw-tooth rete ridges and more frequently exhibit atrophy.[9]

Oral submucous fibrosis (OSMF) is defined as a chronic insidious disease affecting any part of the oral cavity and sometimes pharynx, occasionally preceded and/or associated with vesicle formation and always associated with juxtaepithelial inflammatory reaction followed by fibroblastic change of the lamina propria with epithelial atrophy leading to stiffness of oral mucosa causing trismus and inability to eat. The WHO subsequently defined OSMF as a slowly progressive disease in which the fibrous bands form in the oral mucosa, ultimately leading to severe restriction of movement of the mouth including the tongue.

In 1956, Paymaster first described the precancerous nature of OSMF. In 1972, Pindborg et al. and various groups put forward 5 criteria to prove that OSMF is precancerous. Malignant transformation rate of OSMF was found in the range of 7%-13% depending on the study population.[10]

The development of cancer is a multistep process, where accumulation of genetic defects, followed by clonal selection and expansion of altered cells, ultimately leads

to the development of cancer. Since accumulation of genetic defects can be studied only with changes at molecular level prior to the evident appearance of cellular or clinical changes, detection of these molecular changes would ideally allow earlier diagnosis of high risk states, and therefore help improve prognosis.[11]

Increasing evidence indicates that the unique member of the inhibitor of apoptosis (IAP) protein family, survivin, is not only an essential protein molecule for the regulation of mitosis and apoptotic inhibition but it also plays a role in certain physiological processes as well as in pathological conditions such as carcinogenesis in many human organs/cells. Eight members of the family of IAPs are reported, including X-linked IAP, cIAP1, cIAP2, NAIP (NLR family, apoptosis inhibitory protein), livin, ILP2 (IAP-like protein 2), BRUCE and survivin.[12] The IAP family contains various members that have been shown to inhibit activated caspases.[13] Diffuse expression of survivin has been observed during fetal development but the expression in adult tissues is rare or totally absent. Moreover, survivin expression has been detected in various human cancers including bladder, colon, liver, brain, lung, and prostate. In the majority of cancers studied to date, survivin expression is associated with poor prognosis.[14]

Survivin is a member of the IAP family, encoded as a 16.3 kilodalton protein consisting of 142 amino acids.[15] The proteins are characterized by a domain of about 70 amino acids, termed baculovirus IAPs repeat (BIR). Unlike other IAPs, survivin is small and has only a single N-terminal BIR domain, a long C-terminal alpha-helix coiled region, and forms a stable dimmer in solution.

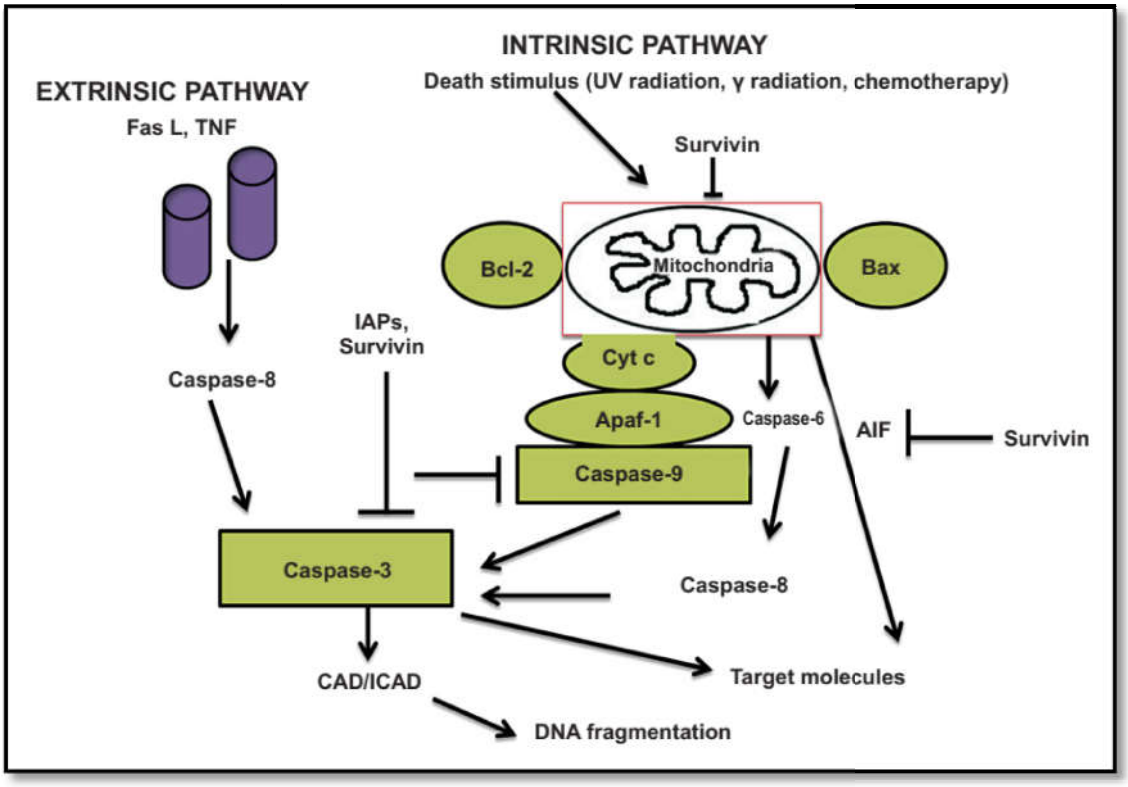


Fig 1: Survivin pathways to apoptosis[12]

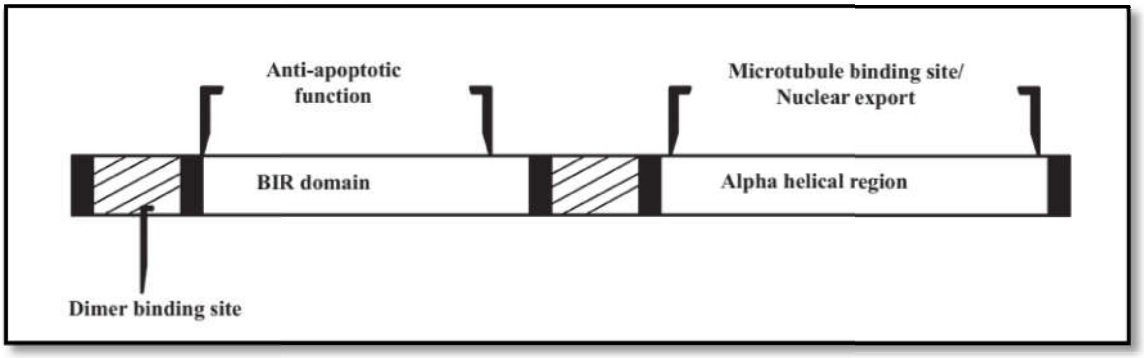


Fig 2: Structure of Survivin Protein[12]

It inhibits apoptosis differently than bcl-2 either by directly or indirectly interfering with caspase-3 and caspase-7 function via its BIR domain. Survivin also neutralizes cell death by interfering with the processing of caspase-9, the upstream inhibitor in the intrinsic pathway of apoptosis.[16] High expression of survivin was observed in the Gap_2 (G_2)/Mitosis (M) phase and a rapid decline was noted in Gap_1 (G_1) phase of cell

cycle. Positive correlation between survivin expression levels with aggressive disease and poor clinical outcome have been established.[12]

The various functions of survivin are broadly classified into two categories:

Mitotic regulation	Cell-death inhibition
Localization to the mitotic apparatus	Inhibition of extrinsic/intrinsic apoptotic pathways
Catastrophic defect of mitosis in survivin –/– embryos	Resistance to apoptosis in transgenic mice
Failed cytokinesis and polyploidy induced by antisense or dominant-negative mutants	Increased sensitivity to apoptosis of survivin +/– mice
Defects in spindle assembly, chromatid separation and spindle-checkpoint activation after antibody microinjection	Spontaneous apoptosis induced by antisense, dominant-negative mutants or ribozyme and association with caspases.

Table 1: Functions of survivin

Survivin expression was found to be localized mainly in the parakeratin/keratin and prickle cell layer of oral leukoplakia cases, with cytoplasmic positivity. Survivin expression was found to be weak in well differentiated squamous cell carcinomas (WDSCCs), in which the keratinocyte showed weak to moderate cytoplasmic survivin expression. All poorly differentiated squamous cell carcinomas (PDSCCs) showed moderate to strong survivin expression and distinct nuclear expression of survivin. The degree of survivin expression was found to increase with increasing grade of malignancy.[13]

Ambrosini et al reported a new human gene encoding a structurally unique IAP apoptosis inhibitor which was designated as survivin. They demonstrated that survivin, while being undetectable in terminally differentiated adult tissue, was prominently expressed in transformed cell lines and in most common forms of human cancers of lung, colon, breast, prostrate, and pancreas, *in vivo*.[17] **Fortugno et al** reported that survivin exists in immunochemically distinct subcellular pools (cytoplasmic and nuclear).[18] **Li et al** reported that the cytoplasmic survivin played a role in cell survival but not in cellular proliferation while nuclear pool of survivin was involved in promoting cell proliferation in most cases.[19]

Lin et al reported that survivin staining was mainly cytoplasmic; it could be found in both oral SCC cells and dysplastic basal and spinous epithelial cells but was rarely noted in normal oral epithelial cells.[20] In the study by **Kim et al**, survivin protein was detected at varying levels in all OSCC cell lines and clinical samples of OSCC in which survivin was predominantly expressed in the proliferating basal cell layer of the tumor mass. On the contrary, survivin expression was not observed in normal gingival keratinocytes.[14]

Observing a high frequency of tumor cells expressing p63 and survivin, **Lauxen et al** highlighted the role of these proteins in the malignant transformation of oral epithelium and suggested that p63 and survivin may constitute attractive targets for cancer therapy in patients with OSCC.[21] No correlation was however found between survivin or p53 expression and clinicopathological parameters by **Khan at al** who concluded that overexpression of these two markers suggests their role in early stages of oral carcinogenesis. Reporting prognostic significance only for the markers survivin and phosphorylated protein kinase strain AK thymoma (pAkt), these authors

concluded that these two markers, connected with cell migration, might have utility as predictors of long-term outcomes for patients with HNSCC.[22]

Pickhard et al reported a correlation between higher cytoplasmic survivin scored and high scores of EGFR expression, and further observed an association between nuclear survivin expression and poor overall survival rate.[23] **Li et al** concluded that survivin might inhibit both synthesis and activation of caspase 3, hence inhibiting cell apoptosis and facilitating eventual development of OSCC.[24]

Considering this background of survivin's role in oral carcinogenesis, this study was undertaken to correlate the immunohistochemical expression of survivin in oral potentially malignant disorders.

Objectives

OBJECTIVES

1. Evaluation of survivin immunoexpression patterns and survivin immunoreactivity in OL, OSMF, OLP and NOE.

2. Comparison of immunoexpression patterns and immunoreactivity of survivin between OL, OSMF, OLP and NOE; between OL and OSMF; between OL and OLP; and between OSMF and OLP and evaluate the significance of survivin as a prognostic marker.

Review of Literature

Ambrosini et al (1997) reported a new human gene encoding a structurally IAP apoptosis inhibitor which was designated as survivin. They demonstrated that survivin, while being undetectable in terminally differentiated adult tissue, was prominently expressed in transformed cell lines and in most common forms of human cancers of lung, colon, breast, prostrate, and pancreas, *in vivo*.[17]

Fengzhi Li et al (1998) reported that a new IAP protein, survivin, was expressed in the G_2/M phase of the cell-cycle in a cycle-regulated manner. At the beginning of mitosis, survivin associates with microtubules of the mitotic spindle in a specific and saturable reaction that is regulated by microtubule dynamics. The results indicated that survivin may counteract a default induction of apoptosis in G_2/M phase.[25]

Ambrosini et al (1998) reported the existence of a potential effector cell protease-1/survivin gene cluster and identified survivin as a new target for disrupting cell viability pathways in cancer. Constitutive or metallothionein induction of effector cell peptidase receptor 1, potentially acting as a survivin antisense, down-regulated endogenous survivin in transformed cells and resulted in increased apoptosis and inhibition of cell proliferation.[26]

Jiang et al (2001) examined the distribution of endogenous levels of survivin in both untreated and Taxol-treated HeLa (Henrietta Lacks) cells, as well as in other tumor-derived and normal cell lines derived from different tissues and reported that during interphase, endogenous survivin in untreated HeLa cells was reproducibly observed localized closely with a pair of tubulin foci on the centrosomes, establishing the role of survivin in mitosis and apoptosis.[27]

Frost et al (2002) evaluated the survivin expression in 73 cervical squamous tissue samples that included 31 normal, 17 low- and 15 high grade squamous intraepithelial lesions and 10 SCCs. Survivin was detected in 22 (71%) of 31 sections of benign cervical squamous mucosa, in 14 (82%) of 17 sections of low grade intraepithelial lesions, in 9 (60%) of 15 sections of high grade intraepithelial lesions, and in 4 (40%) of 10 sections of SCCs. While the nuclear areas of normal cervical mucosa was stained with survivin and cells in the basal layer showed no staining for survivin, staining in other pathological lesions showed cytoplasmic staining predominantly.[28]

Lo Muzio et al (2003) evaluated the survivin expression in 110 samples of paraffined and 10 frozen specimens of primary OSCC, 7 paraffined specimens of lymph node (6 cases) and tissue metastases (1 case) of OSCC. Immunohistochemical staining for survivin was observed in 20– 100% of cancer cells. While the neighboring normal tissues did not express survivin, about 91 cases (82.7%) of oral mucosa cancers positively expressed survivin. Patients with low survivin expression had better survival rates than the group with medium to high survivin expression. It was concluded that survivin expression may identify cases of oral SCC with more aggressive and invasive phenotype.[29]

Lo Muzio et al (2003) evaluated the expression of survivin in 51 samples of which 16 were oral epithelial dysplasias that progressed to invasive OSCCs, 30 cases of oral epithelial dysplasias that did not evolve into OSCC and 5 cases from normal oral mucosa (NOM). Survivin staining was sporadic and weak in the basal and parabasal layers in normal mucosal specimens, detectable in 10 of 30 cases (33%) of precancerous lesions that did not progress to OSCC, 15 of 16 cases (94%) of

precancerous lesions that progressed to OSCC and in 16 of 16 cases (100%) of OSCC specimen.[30]

Tanaka et al (2003) evaluated survivin expression in 71 patients of OSCC and 38 cases of OL with NOM as control. All NOM specimen showed absence or significant down regulation of survivin expression. Among the tumors examined, 41 cases (58%) revealed survivin immunoreaction in the cytoplasm of tumor cells. Fourteen (14) of the 38 OLs (37%) were considered survivin positive. Overall, 37% of the evaluated premalignant lesions showed survivin expression.[31]

Lo Muzio et al (2003) analyzed the survivin expression in 47 samples that included 11 OSCC, 16 OL, and 20 normal oral epithelial (NOE) specimens. Human Papilloma Virus (HPV) positive precancerous lesions and HPV negative OSCCs demonstrated higher levels of survivin, concluding that HPV may have a direct or indirect effect on the regulation of the survivin expression level in oral premalignant and malignant diseases.[32]

Smith et al (2003) performed a systematic review of biomarkers of dysplasia in the oral cavity and reported that the presence of loss of heterozygosity or allelic imbalance at specific loci, survivin and matrix metalloproteinase-9 positivity, and non-diploid deoxyribonucleic acid (DNA) content increase the risk of progression from dysplasia to oral cancer.[33]

Sharma et al (2004) evaluated the anti-apoptotic protein expression in 50 cases of HNSCC and in 19 histopathologically normal tissues and reported that B Cell Lymphoma 2 (Bcl-2), BCL Extra large (Bcl-XL), and survivin were upregulated in tumor tissues in comparison to normal tissues. Expression of Bcl-2 and survivin was

significantly associated with loss of differentiation in tumors and Bcl-XL with nodal metastasis.[34]

Li et al (2005) reported that survivin exists in 2 subcellular pools (cytoplasmic and nuclear) which were consistent with its function in the regulation of both cell viability and cell division. The cytoplasmic survivin played a role in cell survival but not in cellular proliferation while nuclear pool of survivin was involved in promoting cell proliferation in most cases. Survivin had a number of splicing variants, differing in their subcellular localization and functions with respect to cell survival and cell division.[19]

Kim et al (2005) evaluated 281 lymph nodes in 97 patients out of 113 patients with OSCC and reported that lymph node cytokeratin and survivin messenger ribonucleic acid (mRNA) expression had significant effects on OSCC survival rates. They reported that the survivin positive group had a 2.5 times greater OSCC risk than the negative group.[35]

Lo Muzio et al (2005) evaluated 78 cases of OSCC and reported that survivin expression, stage and grade of differentiation are significant to survival and survivin expression may identify cases of OSCC with more aggressive and invasive phenotype.[36]

Lin et al (2005) evaluated survivin expression in 62 cases of epithelial dysplasia and 92 cases of OSCC. Cytoplasmic survivin staining was positive in 97% of epithelial dysplasia cases and in 98% of OSCC cases but there was no evidence of staining in adjacent NOM. They reported that survivin expression is a common feature of OPMD.[20]

Freir et al (2006) studied the immunohistochemical expression of survivin in 251 OSCC tissue sections. High survivin expression was found in 67.3% (169/251) of cases and showed decreased overall survival of patients. They concluded that survivin might be used as a stratification marker to define OSCC patients, who would potentially benefit from radiotherapy.[37]

Jane et al (2006) evaluated the expression of survivin, Bcl-2, and Bcl associated X (Bax) in 38 patients of OSCC and 17 patients of OL and reported that the expression of survivin, Bcl-2, and Bax was found to increase with increased grades of malignancy. Increase in the expression of survivin was statistically more significant.[13]

Fukuda et al (2006) reported that using molecular dissection of genes associated with aberrant proliferation of cancer cells and endothelial cells have identified survivin as a candidate gene responsible for cancer progression and vascular disease and as an attractive molecular therapeutic target for management of oral cancer and oral premalignant conditions.[38]

Pannone et al (2007) evaluated the survivin expression and M-phase promoting factor (MPF) in 32 OSCCs and 17 dysplasias from areas adjacent to OSCC. Survivin and p-34cdc2 expression was positive in all cases of OSCC while cyclinB1 was positive in only 80% of cases. All OPLs associated with OSCC expressed survivin and p-34cdc2 while cyclin B1 was positive in only 70% of cases. It was concluded that MPF and survivin are expressed during early and late phase of oral carcinogenesis.[39]

Knauer et al (2007) demonstrated that nuclear export is essential for the biological activity of survivin and promote the identification of molecular decoys to specifically

interfere with survivin's nuclear export as potential anticancer therapeutics via a newly identified evolutionary conserved Crm1-dependent nuclear export signal (NES) in survivin, thereby confirming an active role of survivin-specific transport inhibitors in anticancer therapeutics.[40]

Preuss et al (2008) evaluated survivin expression in formalin-fixed, paraffin embedded (FFPE) tissue from 106 patients histologically confirmed SCC of the oropharynx. While nuclear expression of survivin was found in 19% of the tumours, cytoplasmic expression was evident in 58% of the cases. This study showed that nuclear accumulation of survivin correlates with HPV-independent carcinogenesis and was an independent predictor of poor survival in patients with OSCC.[41]

Zhou et al (2008) evaluated 82 patients with OSMF and OSCC and demonstrated that 20 of the 40 OSMF cases (50%) showed phosphorylation of survivin threonine 34 and reported that protein phosphorylation is the most important post translational modification that is implemented in the regulation of cell proliferation, differentiation, apoptosis, cytoprotection and cell cycle transitions. They suggested a key role for survivin Thr34 phosphorylation in tumor development and therapy resistance.[42]

Khan et at (2008) evaluated 45 patients (29 OSCC and 16 oral premalignant lesions) of which 9 cases were OL, 2 were OSMF, and 2 were erythroplakia and reported that 21 of 29 (72%) OSCC patients and 7 of 16 (44%) patients with oral premalignant conditions were survivin positive while NOM specimens did not demonstrate survivin expression. They reported that the overexpression of survivin and p53 in premalignant lesions suggest a role in early carcinogenesis.[43]

Oluawadara et al (2009) evaluated 14 cases of OLP, 6 cases of chronic interface mucositis, 4 cases of epithelial dysplasia and 27 cases of OSCC and reported that survivin expression was observed in 64.3% of OLP cases, 50% in cases of chronic interface mucositis, 100% in cases of oral epithelial dysplasia and 96.3% in cases of OSCC. They also suggested that Lck-PI3K/Akt-survivin molecular loop may play an important role in the spectrum of chronic inflammation, autoimmunity, and cancer transformation in OLP.[44]

Chaiyarit et al (2009) evaluated the immunohistochemical expression of survivin and heat shock protein 90 in formalin fixed paraffin embedded archival blocks of 29 OLP cases and 29 NOM specimens. Basal and suprabasal epithelial cells were positive for survivin in NOM. There were 5 cases with 25% to <50% positively stained epithelial cells, 14 cases with 50% to <75% immunostaining and 10 cases were with >75% immunostaining for survivin. Basal and suprabasal epithelial cells were positive for survivin in OLP cases. Infiltrating mononuclear cells in the lamina propria of OLP lesions were positive for survivin. There was one case with no immunostaining, 7 cases with <25% immunostaining, 9 cases with 25% to <50% immunostaining, 8 cases with 50% to <75% immunostaining; and 4 cases were with >75% immunostaining. Significant differences of survivin expression between NOM and OLP were demonstrated.[45]

Lodi et al (2010) measured the mRNA levels by real time polymerase chain reaction method in 15 specimens of normal mucosa, 17 from oral premalignant lesions and 17 from OSCC. All samples of NOM showed survivin expression but samples from premalignant lesions had higher median survivin expression and the highest survivin

expression was noted in the OSCC group. The authors reported a significant statistical difference between the 3 groups.[46]

Zhou et al[9] (2010) evaluated survivin expression in 15 NOE specimens, 50 cases of OSMF which were newly diagnosed without OSCC and 52 OSCC transformed from OSMF. Survivin expression was localized mainly in the basal/parabasal and prickle cell layers in samples of OSMF. About 24 out of 50 OSMF cases (48%) showed cytoplasmic survivin positivity. Two (2) out of 10 early stage (20%) and 9 out of 20 moderately advanced stage (45%) of OSMF were found to exhibit weak survivin expression. Moderate cytoplasmic survivin expression was evident in 65% of the advanced stage OSMF cases. The important role played by survivin during the malignant transformation of OSMF was analyzed and it may provide an indication to early prevention and diagnosis in the progression of OSMF.[15]

Li et al (2012) estimated survivin immunoexpression using enzyme linked immunosorbent assay (ELISA) in 13 OSCC and 13 peritumoral samples of OSCC and 10 NOM samples. Upon analysis of survivin expression using ELISA, tumor tissue showed significantly higher level of survivin expression than in peritumoral tissue, while survivin in normal tissue from tumor-free patients was significantly lower than tumor and peritumoral tissue. It was concluded that survivin may inhibit cell apoptosis and facilitating eventual development of OSCC.[24]

Santarelli et al (2013) analyzed survivin expression in 55 saliva samples OSCC patients and 30 samples from healthy controls, using enzyme immunometric assay. Positive survivin expression was noted in the saliva of 35 of 55 (63.6%) OSCC patients, while 12 of 30 (40%) samples were positive for survivin among the control

group. Salivary survivin proves to be an interesting biomarker for OSCC, but its use as a single marker may be not sufficient for the early diagnosis of OSCC.[47]

Negi et al (2015) studied immunohistochemical expression of survivin in 45 specimens of FFPE tissue blocks, 15 in each of NOE, OL, and OSCC. Survivin positivity was demonstrated in 20% of normal mucosa cases, 53.33% cases of OL, and 80% OSCC. In cases of OED and SCC, a significantly high incidence of survivin expression was found which may be an early event in initiation and progression of OSCC.[48]

Mishra et al (2015) evaluated expression of six survivin isoforms in 4 oral cancer cell lines (AW8507, AW13516, UPCI-SCC040, and UPCI-SCC029B), a dysplastic oral cell line, 75 paired oral tumor and adjacent normal tissues and 12 NOM tissues from healthy individuals by real time polymerase chain reaction (PCR). A significant predominant expression of survivin was observed in all the cell lines in comparison to the minor isoforms of survivin ($p<0.05$). In comparison to normal tissues from healthy individuals, 88% oral cancer cases showed overexpression of survivin wt while survivin ΔEx3 was upregulated in 64% cases. Survivin 3B and survivin 2B showed overexpression in 33% and 76% cases respectively. Overexpression of survivin 2α was observed in 41% cases and survivin 3α in 50% cases. The study indicated that oral cancers overexpress the antiapoptotic survivin variants, which exhibit an association with advanced tumor stage, implying a role for these variants in oral tumorigenesis.[49]

Suganya et al (2016) evaluated 70 neutral-buffered, FFPE biopsy specimens and grouped them into 3 classes: Group 1 (control) – 10 cases of NOM, Group 2 – 50 cases of OLP confirmed histopathologically and Group 3 – 10 cases of OSCC

(positive control). Higher mean positive cells for survivin were recorded in the OSCC group (28.43 mean positive cells), followed by OLP group (16.65 mean positive cells), and NOE group (2.25% mean positive cells). Survivin expression was seen in 95% cases of OLP which was mainly of moderate intensity (46%), followed by mild intensity in 44% of cases.[50]

Gayathri et al (2017) studied the survivin expression in neutral-buffered, FFPE specimens of 30 patients each with OL and OSCC. Expression of survivin was noted in all grades of dysplasia and OSCC and was significant when compared with different degrees of dysplasia and histopathological grades of OSCC. The high expression of survivin was proposed to be related to malignant transformation in OL and poor prognosis in OSCC.[51]

Kulkarni et al (2017) evaluated survivin expression in 43 cases of WDSCC, moderately differentiated squamous cell carcinoma (MDSCC), and PDSCC. PDSCCs demonstrated significantly high survivin expression compared to WDSCC & MDSCC ($p< 0.01$). An increase in the level of survivin expression was noted in OSCC compared to the controls but the use of survivin as a single marker may be not sufficient for the early diagnosis of OSCC.[52]

Li et al (2017) evaluated 45 cases of FFPE tissue specimens including 16 cases of OL with low-moderate epithelial dysplasia, 12 cases of OL with severe epithelial dysplasia, and 17 cases of W/MDSCC. The expression of survivin was gradually increased (normal < OL with low-moderate dysplasia < OL with severe dysplasia < OSCC) during the progression from abnormal cell proliferation to malignant transformation.[53]

Deo et al (2017) evaluated survivin immunoexpression in 10 FFPE samples each of dysplasia, WDSCC, MDSCC, and PDSCC. Of the 10 cases of dysplasia, variable survivin expression was noted in all cases. Both focal nuclear and cytoplasmic staining was observed. Of the 10 cases of dysplasia, 7 cases showed less than 5% of survivin expression, 2 cases showed 5-25% of survivin expression, and 1 case showed 51–75% of survivin expression. Intergroup comparison revealed a statistical significance between dysplasia and MDSCC.[54]

Materials & Methods

Prior to the commencement of the study, ethical approval was obtained from the institutional review board of Best dental science college, Madurai, (Annexure I). Appropriate permissions were obtained from Meenakshi Mission Hospital and Research Centre, Madurai to carry out the immunohistochemistry (IHC) procedures.

SOURCE OF DATA:

This retrospective study was done by retrieving FFPE tissue specimens of OL, OSMF, and OLP from the archives and fresh specimens of NOE in Department of Oral Pathology and Microbiology, Best Dental Science College and Hospital, Madurai.

STUDY SAMPLE SIZE:

The study material comprised of 60 FFPE tissue specimens

1. 15 histopathologically confirmed tissue specimens of OL.

2. 15 histopathologically confirmed tissue specimens of OSMF.

3. 15 histopathologically confirmed tissue specimens of OLP.

4. 15 histopathologically confirmed tissue specimens of NOE.

STUDY SAMPLE GROUP:

The study comprised of 4 groups:

Group I (Cases)

Fifteen (n=15) archival blocks of histopathologically confirmed tissue specimens of OL.

Group II (Cases)

Fifteen (n=15) archival blocks of histopathologically confirmed tissue specimens of OSMF.

Group III (Cases)

Fifteen (n=15) archival blocks of histopathologically confirmed tissue specimens of OLP.

Group IV (Controls)

Fifteen (n=15) fresh samples of NOE.

INCLUSION CRITERIA:

Histopathologically confirmed diagnosis of

1. OL – histopathologically confirmed cases of epithelial dysplasia including CIS
2. OSMF – Different stages of the lesion
3. OLP
4. NOE – derived from fresh samples from department of periodontics

EXCLUSION CRITERIA:

1. Cases of oral squamous cell carcinoma
2. Cases of lichenoid reactions
3. Insufficient case reports.
4. Insufficient tissue samples.

CHEMICAL REAGENTS USED:

1. Primary antibody - 6 milliliter (mL) (Rabbit monoclonal antibody survivin, **Path in situ®**) **(Fig 3).**
2. Secondary antibody (Biogenex Super sensitive Detection system) **(Fig 4).**
3. Tertiary antibody (Biogenex Super sensitive Detection system) **(Fig 5).**
4. 3,3'-Diaminobenzidine (DAB) chromogen (Biogenex Super sensitive Detection system) **(Fig 6).**
5. Methanol (Fischer Scientific India Pvt Ltd).
6. Anhydrous citric acid (Merck Specialties Pvt Ltd).
7. Trisodium citrate dihydrate (Merck Specialties Pvt Ltd) **(Fig 7).**
8. Xylene (Fischer Scientific India Pvt Ltd).
9. Absolute alcohol (Jiangsu Huaxi International Trade Co Ltd, China).
10. Sodium hydroxide (NaOH, Rankem Lab reagent, India).
11. Sodium chloride (NaCl, Qualikems fine chemicals, India).
12. Tris buffer (Fischer scientific India Pvt Ltd **(Fig 8).**
13. Hydrochloric acid (HCl, Qualikems fine chemicals, India).
14. 30% Hydrogen Peroxide (H_2O_2).
15. Hematoxylin (Merck specialties Pvt Ltd).
16. Distyrene Plasticizer Xylene (DPX, Fischer Scientific India Pvt Ltd).

Fig 3: Primary antibody

Fig 4: Secondary antibody

Fig 5: Tertiary antibody

Fig 6: DAB Chromogen

Fig 7: Chemicals for preparation of citrate buffer

Fig 8: Chemicals for preparation of Tris Buffer Saline (TBS)

EQUIPMENTS USED:

1. Magnetic stirrer (Scientific instruments Pvt Ltd) **(Fig 9).**
2. Electronic weighing machine (Citizen, Wensen Pvt Ltd) **(Fig 10).**
3. Potential of Hydrogen (pH) meter (Hanna instruments, Mauritius) **(Fig 11).**
4. Microwave oven **(Fig 12).**
5. Fine tissue paper.
6. Slide rack.
7. Borosil jars.
8. Measuring cylinder
9. Fully automated microtome (Leica RM2125RT) **(Fig no.13)**
10. Slide truft.
11. Polylysine coated slides.
12. Moist chamber.
13. Forceps.
14. Micropipettes (10-100/5-10 microliter) (Perfect micropipettes, India).
15. Refrigerator.
16. Light microscope (Olympus BX53®) **(Fig no.14)**.

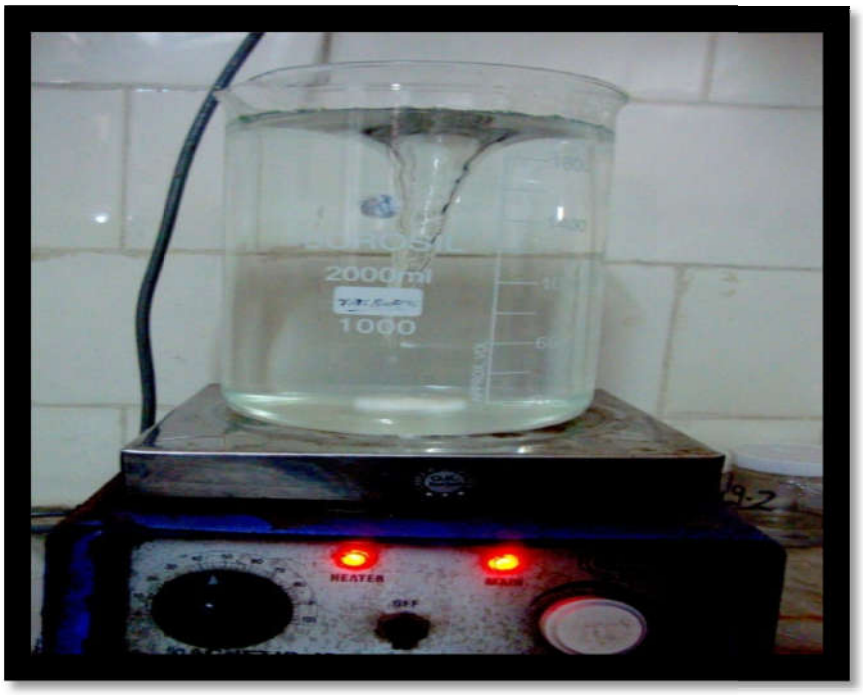


Fig 9: Magnetic stirrer

Fig 10: Weighing machine

Fig 11: pH meter

Fig 12 : Microwave oven

Fig no.13: Fully automated microtome

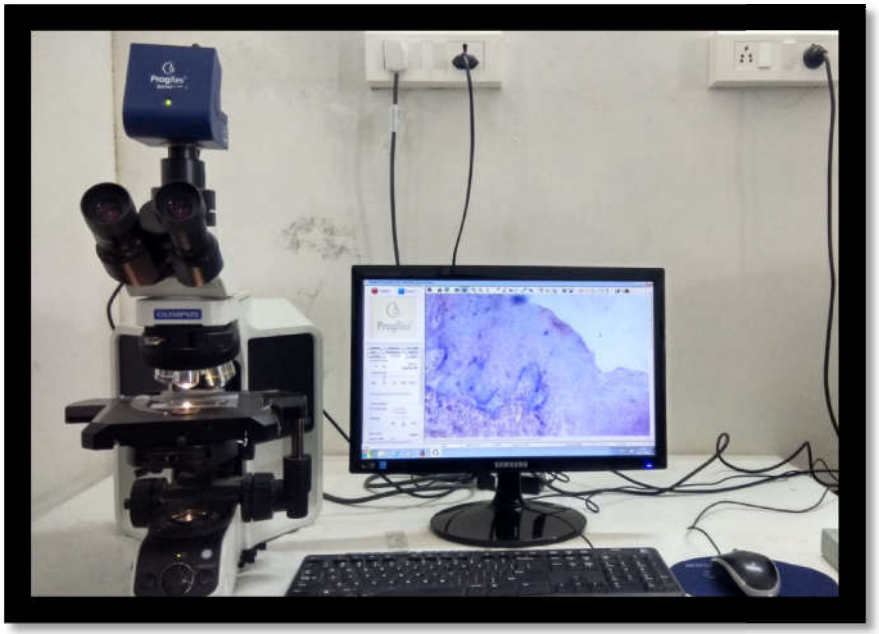


Fig no 14: Olympus BX53 Light microscope and photomicrography done with Prog Res Speed XT Core 3.

METHODOLOGY

Prior to commencement of the IHC staining procedure, citrate buffer and Tris Buffer Saline (TBS) were prepared.

Preparation of citrate buffer [pH 6.0, 1 liter (L)]

1. Weigh 0.18 gram (g) anhydrous citric acid.
2. Weigh and add 2.1 g sodium citrate.
3. Add 4-8 drops of NaOH.
4. Add distilled water in measuring cylinder up to 1000 mL.
5. Mix well by magnetic stirrer.
6. Control pH by NaOH/HCl.

Preparation of TBS (pH 7.4 to 7.6, 2 L)

1. Weigh NaCl 17.2 g.
2. Weigh and add tris buffer 12.0 g.
3. Add 7.0 mL of 1 normality (N) HCl (To make 1N HCl, take 37 mL HCl and distilled water up to 1 L).
4. Add distilled water up to 1000 mL and mix well by magnetic stirrer.
5. Control pH by adding drop by drop 1N HCl.

IMMUNOHISTOCHEMICAL STAINING PROCEDURE

From the clinically and histopathologically confirmed FFPE blocks, sections that were 4 microns thick were cut and stained with routine Hematoxylin and Eosin staining. The histological features of OL, OSMF, and OLP were analyzed under light microscopy. Cases that met the histologic criteria of OL, OSMF, and OLP were selected for the immunohistochemical analysis. Later these selected slides were subjected to IHC procedure. IHC procedures were carried out in Meenakshi Mission Hospital and Research Centre, Madurai, as per the step-by-step procedure described below:

STEP 1: COATING OF SLIDES

Take thin section of tissues on polylysine-coated slides, one day prior to the IHC staining procedure.

STEP 2: DEPARAFFINISATION AND REHYDRATION OF TISSUE

1. Xylene, two changes [5 minute (min) each, at room temperature].
2. Acetone, one change (3 min).
3. Absolute alcohol (3 min).
4. Running tap water (15 min).
5. Leave the section in water until the next step.

STEP 3: ANTIGEN RETRIEVAL

1. Put the slides with the truft in the citrate buffer jar.

2. Microwave it at high power for 30 min or 15 min high power and 10 min low power.

3. Bring the solution at room temperature.

4. Discard the citrate buffer.

5. One wash by TBS.

STEP 4: ENDOGENOUS PEROXIDISE BLOCKAGE

1. Make 4% H_2O_2 (96 mL methanol + 4 mL 30% H_2O_2).

2. Put the slides in truft and leave in 4% H_20_2 for 30 min at room temperature.

3. TBS wash (3 changes) 5 min each.

STEP 5: PRIMARY ANTIBODY

1. Take out the slides from the truft one by one.

2. Wipe off the excess liquid by fine tissue paper.

3. Mark the tissues by wax pens.

4. Put the slide in moist chamber.

5. Apply one drop of primary antibody to each section.

6. Incubate at room temperature for 1 hour.

7. 3 changes of TBS (5 min each).

STEP 6: SECONDARY ANTIBODY

1. Wipe off the excess liquid.
2. Put the slides in moist chamber and apply secondary antibody.
3. Incubate at room temperature for 30 min.
4. 3 changes of TBS (5 min each).

STEP 7: LINK ANTIBODY/TERTIARY ANTIBODY

1. Wipe off the excess liquid.
2. Add link antibody and incubate at room temperature for 30 min.
3. 3 changes of TBS (5 min each) and keep the slides in TBS.

STEP 8: DAB CHROMOGEN

1. Wipe off excess liquid.
2. Apply the DAB chromogen.
3. See under the microscope for colour.
4. If satisfactory staining, remove the DAB.
5. Immerse in distilled water to stop the reaction.

STEP 9: COUNTER STAINING (HEMATOXYLIN)

1. Make 1 to 3 dips in hematoxylin for counter staining.
2. Wash under running water.

STEP 10: DEHYDRATION OF TISSUE AND MOUNTING

1. Absolute alcohol (1 change).
2. Acetone (1 change).
3. Xylene (2 changes).
4. Take out the slides from xylene and mount.

EVALUATION METHODS:

All the slides were viewed using an Olympus® BX53 light microscope and compared with their respective Hematoxylin and Eosin sections. Photomicrography of the IHC stained slides were done with ProgRes Speed XT Core 3® **(Fig 14).** To minimize the errors while interpretation, two observers examined the IHC stained slides by means of light microscope. All the immunohistochemically stained slides from the study groups I, II, III, and IV were evaluated for the expression of survivin. Survivin immunopositivity was assessed by the presence of a brown colour immunostaining of the nucleus and cytoplasm.

Evaluation of survivin immunoexpression patterns

The pattern of survivin expression in all the groups was recorded based on their localization as cytoplasmic, nuclear, and both cytoplasmic and nuclear expression.

The intensity of staining was estimated based on the criteria followed by Tanaka et al[31] and Muzio et al [20]:

- Score 0 : No staining
- Score 1 : Mild staining intensity
- Score 2 : Moderate staining intensity

- Score 3 : Strong staining intensity

The percentage of survivin immunopositivity was categorized based on the criteria adopted by Tanaka et al[31] and Muzio et al [20]:

The percentage of survivin immunopositive cells was estimated and graded, in five random fields, on a scale of 0-4 as follows:

- 0 points: No immunopositive cells
- 1 points: <10% immunopositive cells
- 2 points: 10% to 29% immunopositive cells
- 3 points: 30% to 59% immunopositive cells
- 4 points: 60% to 100% immunopositive cells

Evaluation of survivin immunoreactivity

The immunoreactivity of survivin in all the groups was assessed semi-quantitatively by calculating the immunoreactive score (IRS) as follows:

IRS = Percentage of immunopositive cells (A) x Intensity of immunostaining (B)

The IRS score was translated into survivin immunoreactivity as follows

- 0-1: Negative
- 2-3: Mild
- 4-8: Moderate
- 9-12: Strongly positive

STATISTICAL ANALYSIS:

All the relevant clinical, histopathological and immunohistochemical data were entered in Microsoft excel. Data analysis was done with the help of computer and subjected to appropriate statistical analysis using **Statistical Package for Social Sciences (SPSS Inc., Chicago, IL, version 22.0 for Windows).**

Using this software, frequencies and percentages were calculated for qualitative variables. Chi Square Test was employed to evaluate the gender distribution among different study groups (OL, OSMF, OLP and NOE). Fisher's Chi square test was opted to compare the variance in distribution, intensity of staining, percentage of immunopositivity and assess pair wise comparison between the groups namely, OL and OSMF, OL and OLP, and OSMF and OLP. A 'p' value less than 0.05 denotes significant relationship. ($p > 0.05$ – not significant, $p<0.05$ – significant, $p<0.01$ – highly significant, $p<0.001$ – very highly significant).

Results

Clinical characteristics of the study sample

The study group comprised of 60 cases which includes 15 samples of OL, 15 samples of OSMF, 15 samples of OLP, and 15 samples of NOE. The mean age of the patients included in the study was 43 years and ranged from 23 to 67 years. Majority of these cases were from buccal mucosa (n=47, 78%). Other samples were derived from gingiva (n=17, 28.3%), lip (n=1, 1.7%) and tongue (n=1, 1.7%). The clinical characteristics of the pathological conditions included in the analysis are presented in **Table 2**.

All the samples (100%) of OL were obtained from men. The patient's mean age was 48.5 years and ranged from 32 to 67 years. Majority of these cases were from buccal mucosa (n=12, 80%). Other sites reported were gingiva (n=2. 13.3%) and lip (n=1, 6.7%).

Of the 15 OSMF, 8 samples (53.3%) were from males and 7 (46.7%) from females. The mean age of these patients was 48.2 years ranging from 27 to 65 years. All the samples (100%) were obtained from buccal mucosa.

For the OLP study group, 6 (40%) males and 9 (60%) females with a mean age of 39.9 years ranging from 24 to 56 years were selected. Fourteen (14) samples (93.3%) were obtained from buccal mucosa while a single sample (6.7%) was from tongue.

The NOE control group includes 7 (46.7%) males and 8 (53.3%) females with a mean age of 35.2 years ranging from 23 to 52 years. All samples (100%) were obtained from the gingiva.

Comparison of IRS among OL, OSMF, OLP and NOE

Table 3A-B/Graph 1 compares IRS among OL, OSMF, OLP, and NOE. Moderate immunoreactivity was seen in maximum number of samples. The NOE was completely negative for survivin. The immunoreactivity ranged from mild (40%) to moderate (40%) for OL. In case of OSMF, 40% showed negative immunoreactivity, while the remaining samples showed mild (20%) to moderate (40%) expression. The majority of OLP samples showed moderate immunoreactivity (80%), though 13.3% of them were negative. Strong immunoreactivity was seen in 2 samples (3.3%) which include 1 case each of OL and OLP. Statistically high significant difference was found among all the 5 groups (P=0.01).

Pair wise comparison of IRS between OL and OSMF showed that the immunoreactivity for survivin was comparatively more for OL with most cases showing mild (40%) to moderate (40%) expression, along with strong expression in a single case (6.7%). While the IRS for OSMF was moderate in 40% cases with the rest showing negative (40%) to mild (20%) expression. There was no statistically significant difference between the two groups (p = 0.261).

Comparison of IRS between OL and OLP revealed a higher value for OLP with about 80% samples exhibiting moderate immunoreactivity, whereas OL showed mild (40%) to moderate (40%) reaction for survivin. A statistically significant difference was noted between OL and OLP (p = 0.05). Similarly, when OLP was compared with OSMF, a considerably higher immunoreactivity was observed for OLP with around 80% samples demonstrating moderate expression. On the other hand, OSMF had moderate score for only 40% of cases along with mild (20%) to negative (40%)

immunoreaction. This difference in IRS between OSMF and OLP was also found to be statistically significant (p = 0.05).

Comparison of intracellular stain location in epithelial cells among OL, OSMF, OLP and NOE

Table 4A-B/Graph 2 compares the intracellular stain location among OL, OSMF, OLP, and NOE. The NOE showed absence of survivin expression however a negligible amount of cells had nuclear expression in few cases. Nuclear staining for survivin was noted in most of the study groups among which OSMF had maximum nuclear positivity (86.7%). Both Cytoplasmic and nuclear expression was found in only few cases of OL (6.7%), OSMF (13.3%) and OLP (20%). Cytoplasmic expression was found in the least number of samples, OL (20%) and OLP (13.3%). A highly significant difference was found statistically among all the 4 groups (P=0.001).

Comparison of intracellular survivin expression patterns between OL and OSMF demonstrated a stronger nuclear expression for OSMF (86.7%) along with both nuclear and cytoplasmic expression in 13.3% samples. Although majority of OL samples exhibited nuclear positivity (73.3%); some cases had expression only in the cytoplasm (20%). However, this difference did not show any significance statistically (P=0.173). Likewise, comparison between OL and OLP also did not produce any significant difference (P=0.535). Both the study groups exhibited nuclear positivity in most cases [OL (73.3%; OLP (66.7%)] with relatively small number of samples showing cytoplasmic [OL (20%); OLP (13.3%)] and nuclear – cytoplasmic expression [OL (6.7%); OLP (20%)].

When the intracellular survivin expression patterns were compared for OSMF and OLP, it was noted that nuclear positivity was higher for OSMF (86.7%) compared to OLP (66.7%). Cytoplasmic expression was seen only in OLP (13.3%) but both cytoplasmic and nuclear expression was found in OSMF (13.3%) and OLP (20%). However, there was no statistically significant difference between the two groups (P=0.273).

Comparison of survivin distribution in the epithelium among OL, OSMF, OLP and NOE

Table 5A-B/Graph 3 compares distribution of survivin in the epithelium of OL, OSMF, OLP, and NOE. Survivin expression was not seen in most samples (86.7%) of NOE, however, there was mild expression in the basal cells of few samples (13.3%). Overall, it was expressed predominantly in the basal and parabasal layers in all the study samples [OL (60%); OSMF (86.7%) and OLP (100%)]. Nevertheless, it was found only in the basal layer of the epithelium in some cases of OL (33.3%) and OSMF (13.3%). Statistical significance across the groups was confirmed to be highly significant (p = 0.01).

Comparison of survivin expression across the layers of epithelium between OL and OSMF showed that it was expressed mainly in the basal and parabasal layers of OSMF (86.7%) compared to OL (60%). The expression of this immunomarker was prominent only in the basal cells of OL in almost 33.3% samples while it was found in only 13.3% of OSMF. However, there was no significant difference between the two groups (P = 0.22). Likewise, comparison of OL with OLP revealed that the marker was distributed predominantly in the basal and parabasal layers of OLP (100%) which was significantly greater than OL (60%) (P=0.02). On the other hand,

when OSMF was compared with OLP, there was no significant difference in their distribution pattern (p = 0.14). Both the study groups exhibited immunoexpression chiefly in the basal and parabasal layers [OSMF (86.7%); OLP (100%)].

Comparison of percentage of immunopositive cells among OL, OSMF, OLP and NOE

Table 6A-B/Graph 4 compares the percentage of survivin immunopositive cells across OL, OSMF, OLP, and NOE. Majority of NOE samples (86.7%) were negative for survivin, though relatively few cells (<10%) showed mild survivin expression in 13.3% of these samples. OL had immunopositivity in the range of <10% (46.7%) and 10-29% (46.7%) but a single case (6.7%) demonstrated greater positivity 30-59%. Most cases (53.3%) of OSMF had expression in only <10% cells and the rest 46.7% cases had survivin expression in 10-29% of epithelial cells. The number of immunopositive cells was higher for OLP with 80% samples exhibiting reaction in 10 -29% cells. Statistically, highly significant difference was noted among all the 4 groups (p = 0.01).

Comparison of percentage of immunopositive cells between OL and OSMF did not show any significant differences (p = 0.22). However, there was a significant difference in the number of immunopositive cells between OSMF and OLP with the latter showing greater expression (P = 0.05). In spite of having greater percentage of survivin expression in OSMF, there was no statistically significant difference with OL (P = 0.13).

Comparison of staining intensity among OL, OSMF, OLP and NOE

Table 7A-B/Graph 5 compares the intensity of survivin staining in OL, OSMF, OLP, and NOE. Mild expression of survivin was seen in few basal cells of NOE in 13.3% samples, even though majority of these tissues (86.7%) showed complete negativity. OL expressed survivin strongly in most cases (46.7%) followed by moderate intensity (33.3%). The samples of OSMF displayed mild (46.7%) to moderate intensity (40%), while a moderate (60%) to strong intensity (26.7%) was observed in OLP. Statistically, highly significant difference was noted between all the 4 groups ($p = 0.01$). Intergroup comparison among the study samples did not yield any statistically significant difference.

Summary of intra- and intergroup analysis of the study samples

Based on the statistical analysis, high significance for survivin expression was noted between the OPMD and the NOE in all five categories. However, there was no significant difference between OL, OSMF and OLP in intracellular stain location and intensity. Significant difference was noted between the groups for IRS, layered distribution of survivin expression and mean immunopositivity. The intergroup comparison and outcome of the analysis between the study groups is summarized in **Table 8**.

Key: "p" is level of significance, ($p > 0.05$ - Not significant; $p < 0.05$ – Significant; $p < 0.01$ - Highly significant; $p < 0.001$ - Very highly significant).

Tables and Graphs

Table 2

Clinical characteristics of each pathological condition included in the analysis

Category		OL		OSMF		OLP		NOE	
		n	%	n	%	n	%	N	%
Sex	Male	15	100%	8	53.3%	6	40%	7	46.7%
	Female	0	0%	7	46.7%	9	60%	8	53.3%
Age	20-40	4	26.7%	5	33.3%	8	53.3%	10	66.7%
	41-60	9	60%	8	53.3%	7	46.7%	5	33.3%
	61-80	2	13.3%	2	13.3%	0	0	0	0
Site	Buccal mucosa	12	80%	15	100%	14	93.3%	0	0
	Gingiva	2	13.3%	0	0	0	0	15	100%
	Lip	1	6.7%	0	0	0	0	0	0
	Tongue	0	0	0	0	1	6.7%	0	0

Table 3A

Comparison of immunoreactivity score among OL, OSMF, OLP and NOE

Groups	N	Immunoreactivity scores			
		Negative	Mild	Moderate	Strong
OL	15	2 (13.3%)	6 (40%)	6 (40%)	1 (6.7%)
OSMF	15	6 (40%)	3 (20%)	6 (40%)	0 (0%)
OLP	15	2 (13.3%)	0 (0%)	12 (80%)	1 (6.7%)
NOE	15	15 (100%)	0 (0%)	0 (0%)	0 (0%)
Total	60	25 (41.7%)	9 (15%)	24 (40%)	2 (3.3%)
Significance P Value		0.01 (**HS**)*			

Table 3B

Intergroup comparison of study samples based on IRS score

Intergroup comparison	Significance
OL vs. OSMF	0.261 (NS)*
OL vs. OLP	0.05 (**S**)*
OSMF vs. OLP	0.05 (**S**)*

**Chi Square Test*

Table 4A

Comparison of intracellular stain location among OL, OSMF, OLP and NOE

Groups	N	Stain location patterns			
		No expression	Cytoplasmic	Nuclear	C + N
OL	15	0 (0%)	3 (20%)	11 (73.3%)	1 (6.7%)
OSMF	15	0 (0%)	0 (0%)	13 (86.7%)	2 (13.3%)
OLP	15	0 (0%)	2 (13.3%)	10 (66.7%)	3 (20%)
NOE	15	13 (86.7%)	0 (0%)	2 (13.3%)	0 (0%)
Total	60	13 (21.7%)	5 (8.3%)	36 (60%)	6 (10%)
Significance P Value		0.001 (**VHS**)*			

Table 4B

Intergroup comparison of study samples based on intracellular stain location

Intergroup comparison	Significance
OL vs. OSMF	0.173 (NS)*
OL vs. OLP	0.535 (NS)*
OSMF vs. OLP	0.273 (S)*

**Chi Square Test*

Table 5A

Comparison of of survivin distribution in the epithelium of OL, OSMF, OLP and NOE

Groups	N	Distribution of survivin immunoreaction			
		No expression	Basal	Basal and Parabasal	Entire Thickness
OL	15	0 (0%)	5 (33.3%)	9 (60%)	1 (6.7%)
OSMF	15	0 (0%)	2 (13.3%)	13 (86.7%)	0 (0%)
OLP	15	0 (0%)	0 (0%)	15 (100%)	0 (0%)
NOE	15	13 (86.7%)	2 (13.3%)	0 (0%)	0 (0%)
Total	60	13 (21.7%)	9 (15%)	37 (61.7%)	1 (1.7%)
Significance P Value		0.01 (**HS**)*			

Table 5B

Intergroup comparison of the study samples based on stain distribution

Intergroup comparison	Significance
OL vs. OSMF	0.22 (NS)*
OL vs. OLP	0.02 (**S**)*
OSMF vs. OLP	0.14 (NS)*

**Chi Square Test*

Table 6A

Comparison of percentage of survivin immunopositivity among OL, OSMF, OLP and NOE

Groups	N	Percentage of survivin immunopositive cells				
		Nil	<10%	10-29%	30-59%	60-100%
OL	15	0 (0%)	7 (46.7%)	7 (46.7%)	1 (6.7%)	0 (0%)
OSMF	15	0 (0%)	8 (53.3%)	7 (46.7%)	0 (0%)	0 (0%)
OLP	15	0 (0%)	2 (13.3%)	12 (80%)	1 (6.7%)	0 (0%)
NOE	15	13 (86.7%)	2 (13.3%)	0 (0%)	0 (0%)	0 (0%)
Total	60	13 (21.7%)	19 (31.7%)	26 (43.3%)	2 (3.3%)	0 (0%)
Significance P Value		0.01 (**HS**)*				

Table 6B

Intergroup comparison of the study samples based on the percentage of survivin immunopositive cells

Intergroup comparison	Significance
OL vs. OSMF	0.58 (NS)*
OL vs. OLP	0.13 (NS)*
OSMF vs. OLP	0.05 (**S**)*

**Chi Square Test*

Table 7A

Comparison of staining intensity among OL, OSMF, OLP and NOE

Groups	N	Staining Intensity Scores			
		Negative	Mild	Moderate	Strong
OL	15	0 (0%)	3 (20%)	5 (33.3%)	7 (46.7%)
OSMF	15	0 (0%)	7 (46.7%)	6 (40%)	2 (13.3%)
OLP	15	0 (0%)	2 (13.3%)	9 (60%)	4 (26.7%)
NOE	15	13 (86.7%)	2 (13.3%)	0 (0%)	0 (0%)
Total	60	13 (21.7%)	14 (23.3%)	20 (33.3%)	13 (21.7%)
Significance P Value		0.01 (**HS**)*			

Table 7B

Intergroup comparison of the study samples based on intensity of staining

Intergroup comparison	Significance
OL vs. OSMF	0.10 (NS)*
OL vs. OLP	0.34 (NS)*
OSMF vs. OLP	0.13 (NS)*

**Chi Square Test*

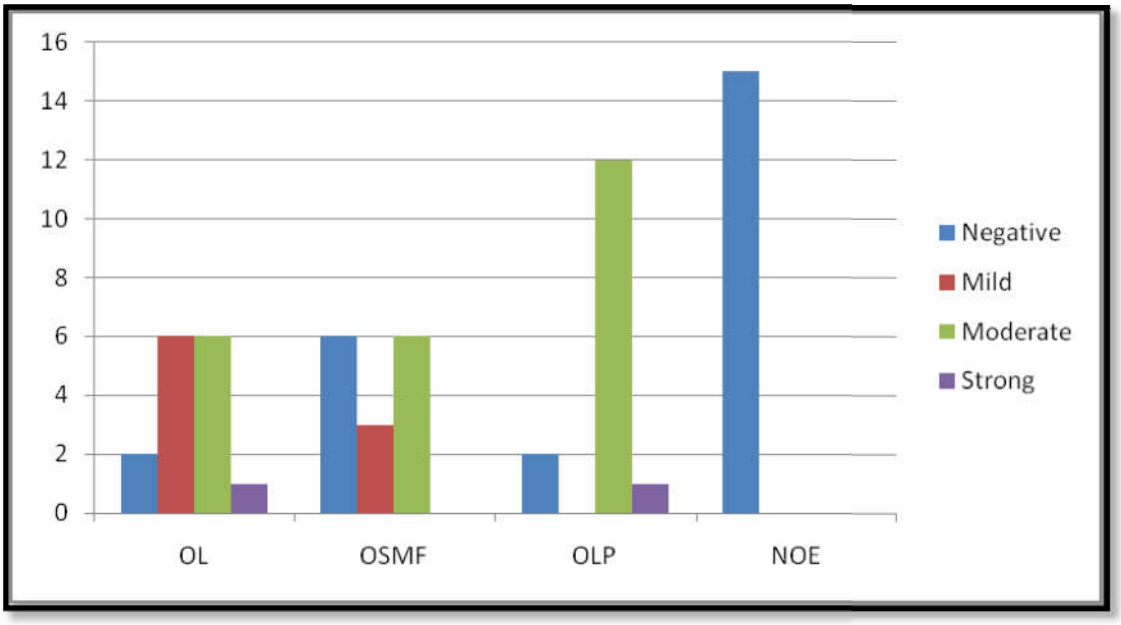
Table 8

Summary tabulation

Category/Groups	All 3 groups	OL vs.OSMF	OL vs.OLP	OSMF vs.OLP
IRS score	**HS**	NS	**S**	**S**
Intracellular Stain location	**HS**	NS	NS	NS
Layer distribution	**HS**	NS	**S**	NS
Percentage of Immunopositivity	**HS**	NS	NS	**S**
Intensity of staining	**HS**	NS	NS	NS

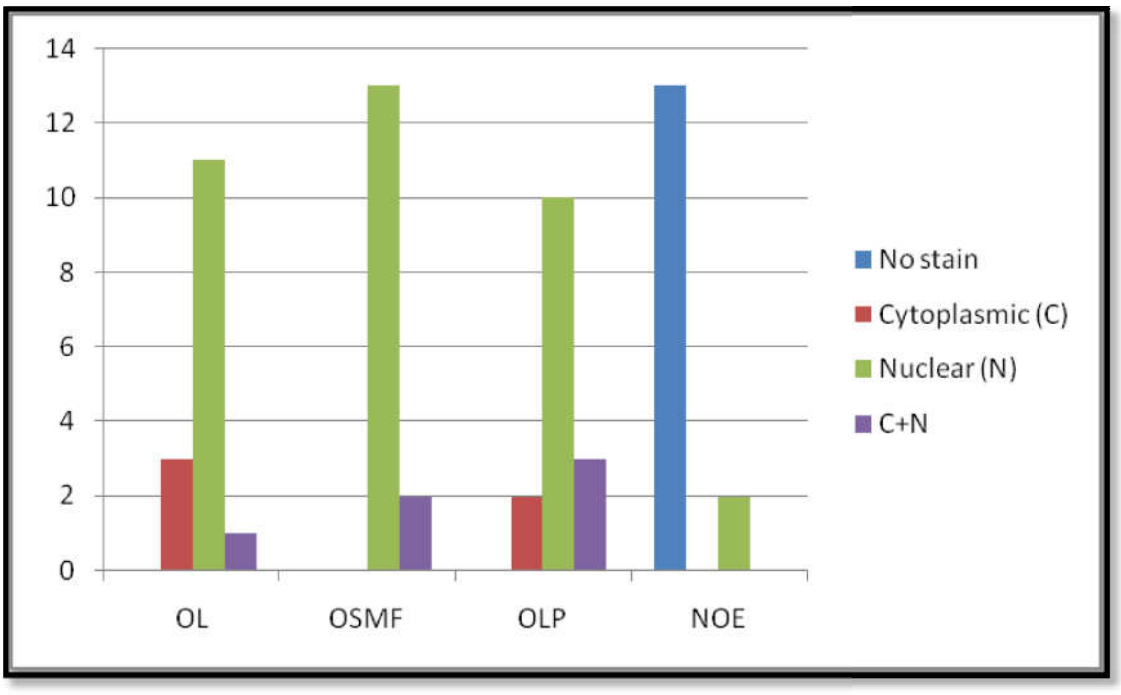
Graph 1

Comparison of immunoreactivity score (IRS) among OL, OSMF, OLP and NOE



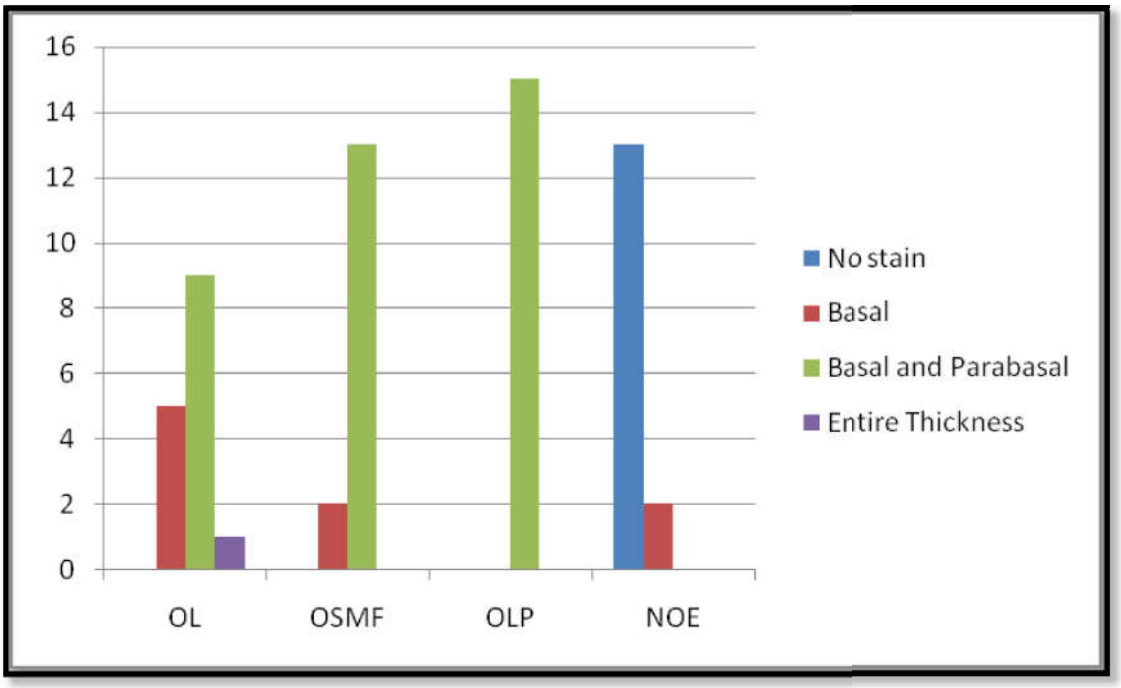
Graph 2

Comparison of intracellular stain location in OL, OSMF, OLP and NOE



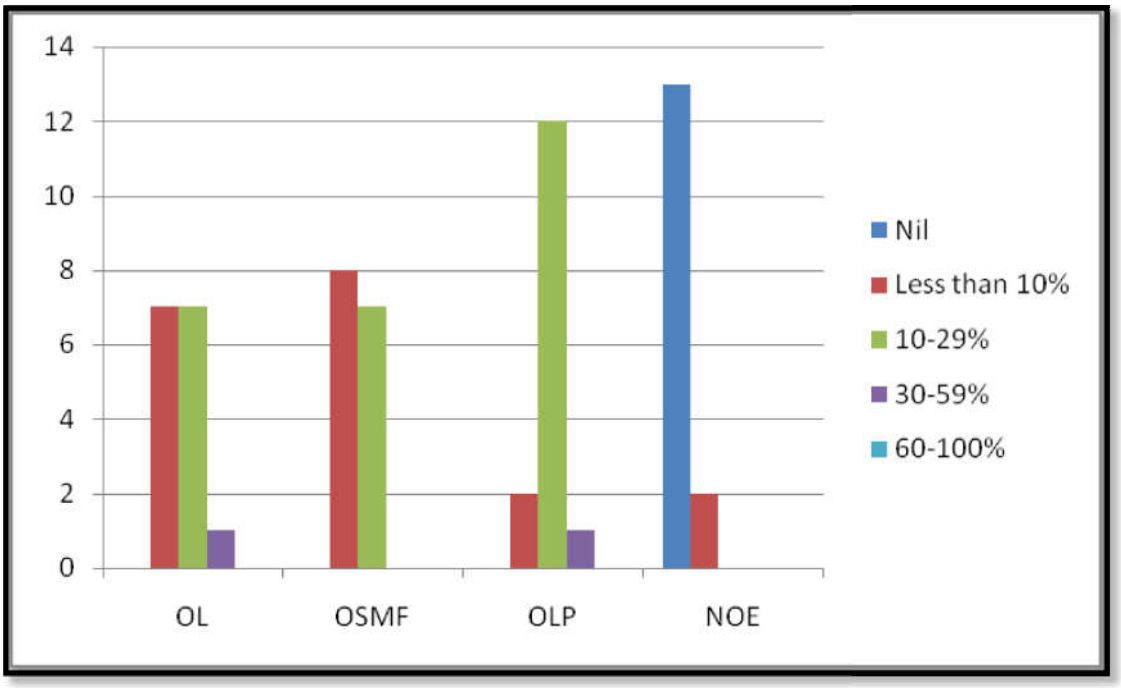
Graph 3

Comparison of survivin distribution in epithelium among OL, OSMF, OLP and NOE



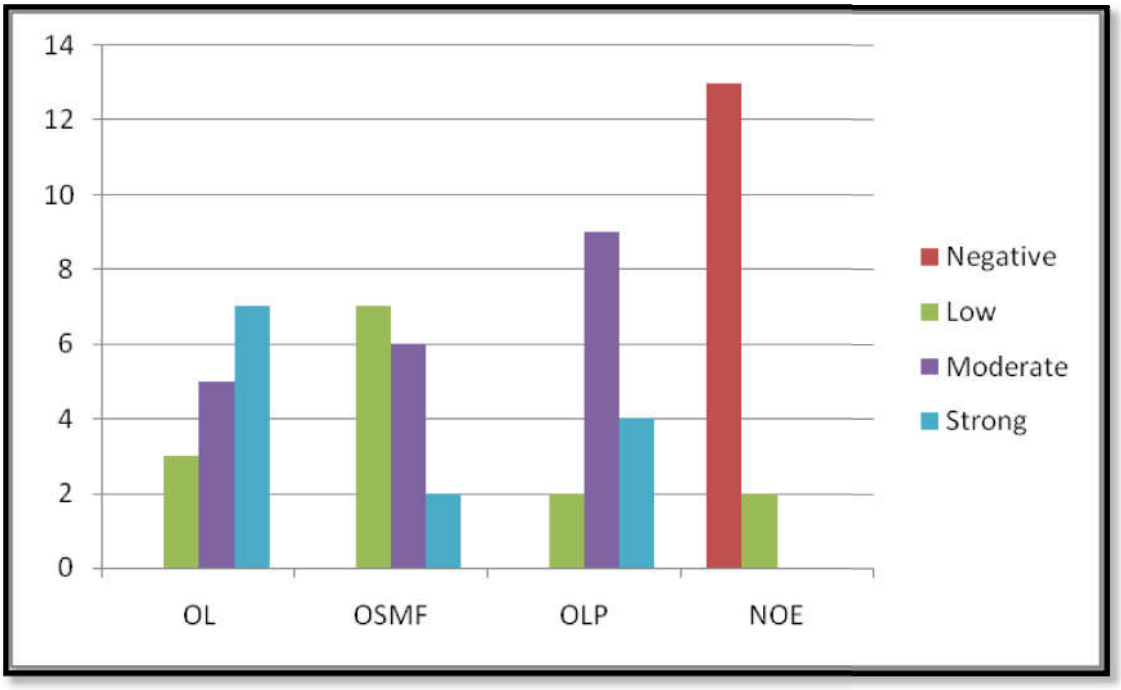
Graph 4

Comparison of percentage of survivin immunopositive cells among OL, OSMF, OLP and NOE



Graph 5

Comparison of staining intensity among OL, OSMF, OLP and NOE



Color plates

Color plate 15 - Oral Leukoplakia

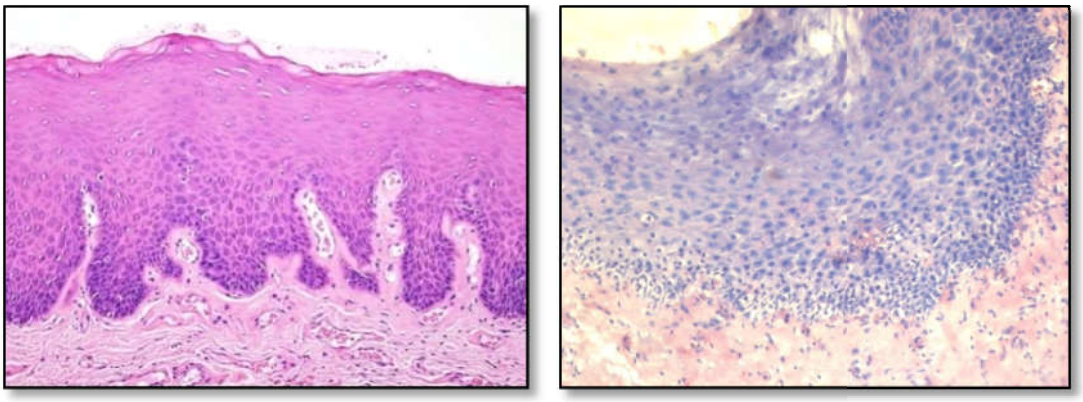


Fig 15A and 15B: Oral leukoplakia showing dysplastic changes in the epithelium and underlying connective tissue (H&E, 40X; 100X)

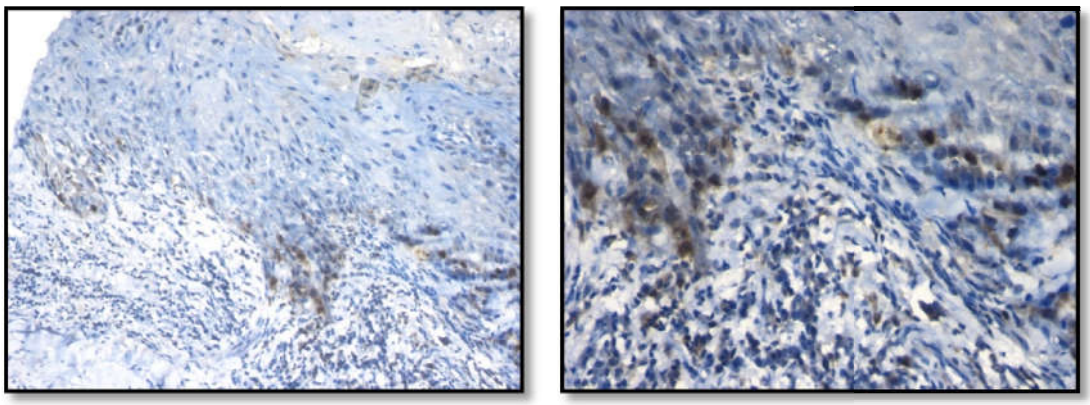


Fig 15C and 15D: Oral Leukoplakia showing survivin immunopositivity in the basal and suprabasal areas (100X; 200X)

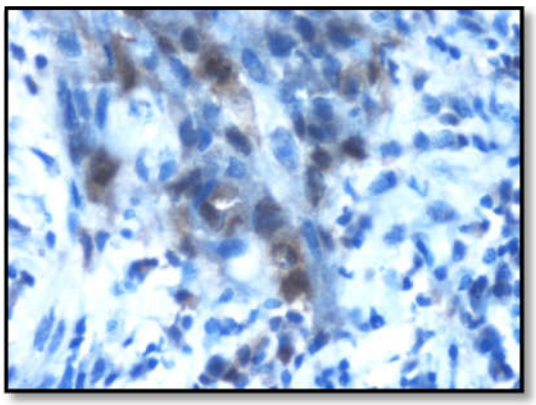


Fig 15E: Oral Leukoplakia showing nuclear and cytoplasmic survivin immunopositivity (400X)

Color plate 16 - Oral Submucous Fibrosis

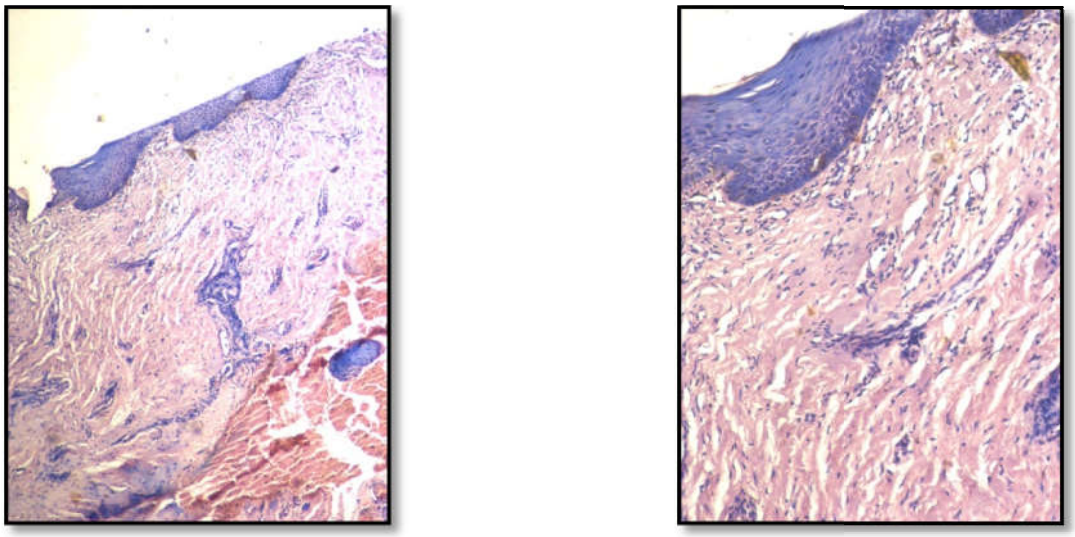


Fig 16A and 16B: Oral Submucous Fibrosis showing surface epithelium and underlying fibrous collagen bundles in the connective tissue ((H&E, 40X; 100X)

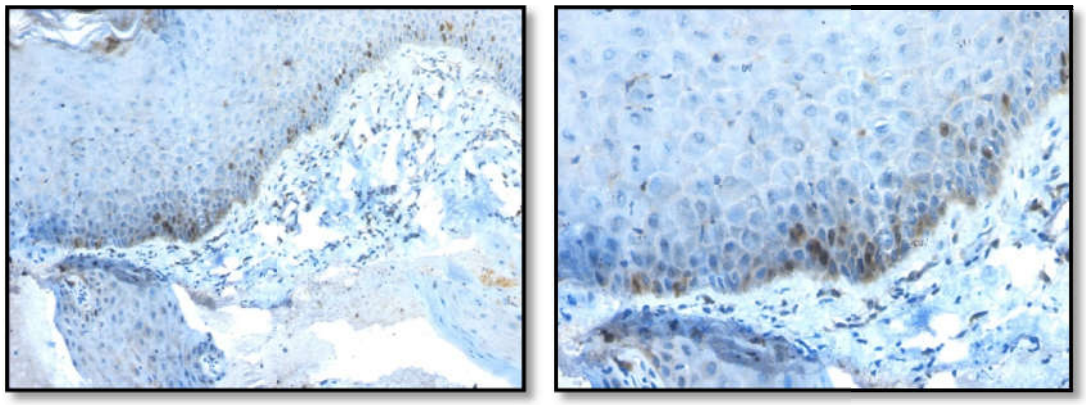


Fig 16C and 16D: Oral Submucous Fibrosis demonstrating positive survivin immunoreactivity in the basal and suprabasal areas (100X; 200X)

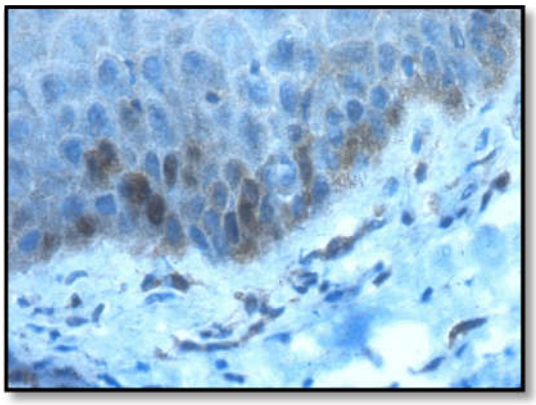


Fig 16E: Oral Submucous Fibrosis demonstrating positive survivin immunoreactivity in the nuclear and cytoplasmic compartments (400X)

Color plate 17 - Oral Lichen Planus

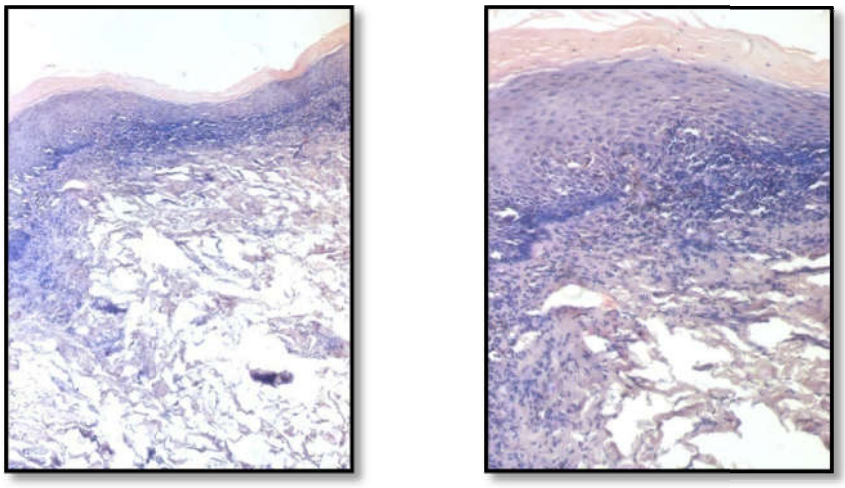


Fig 17A and 17B: Oral Lichen Planus showing surface epithelium with basal layer degeneration and lymphocytic infiltration in the subepithelial connective tissue (H&E, 40X; 100X)

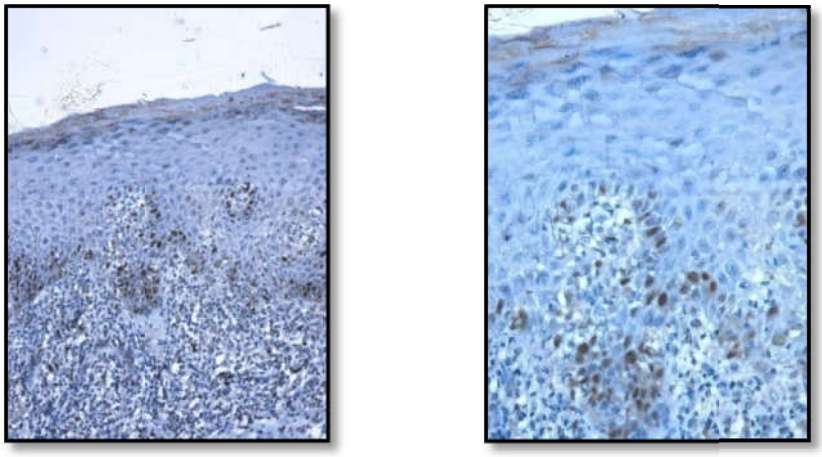


Fig 17C and 17D: Oral Lichen Planus showing positive survivin immunoreactivity in the basal and suprabasal areas (100X; 200X)

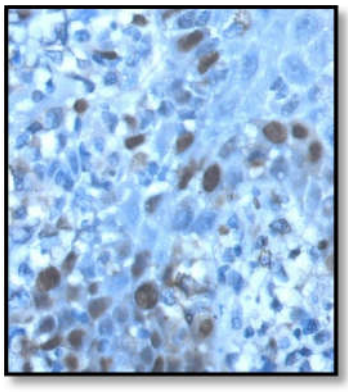


Fig 17E: Oral Lichen Planus showing nuclear survivin immunoreactivity (400X)

Color plate 18 - Normal Oral Epithelium

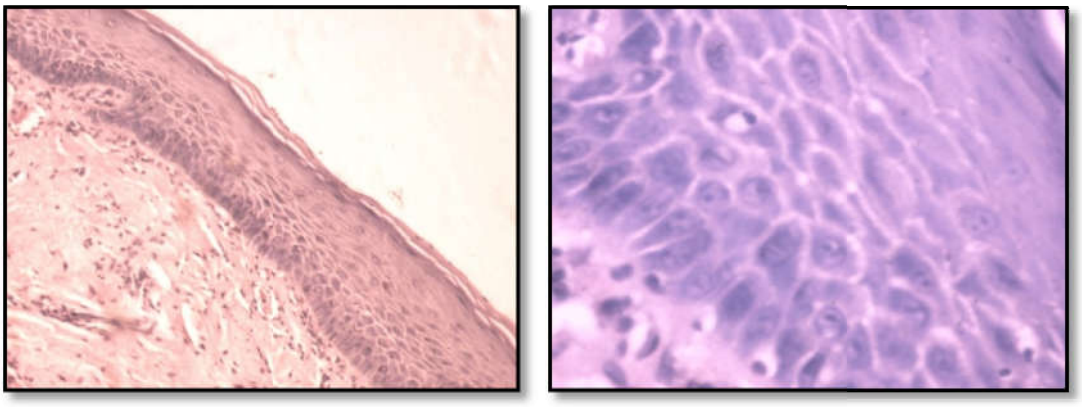


Fig 18A and 18B: Normal oral epithelium showing epithelial lining and underlying fibrous connective tissue (H&E, 100X; 400X)

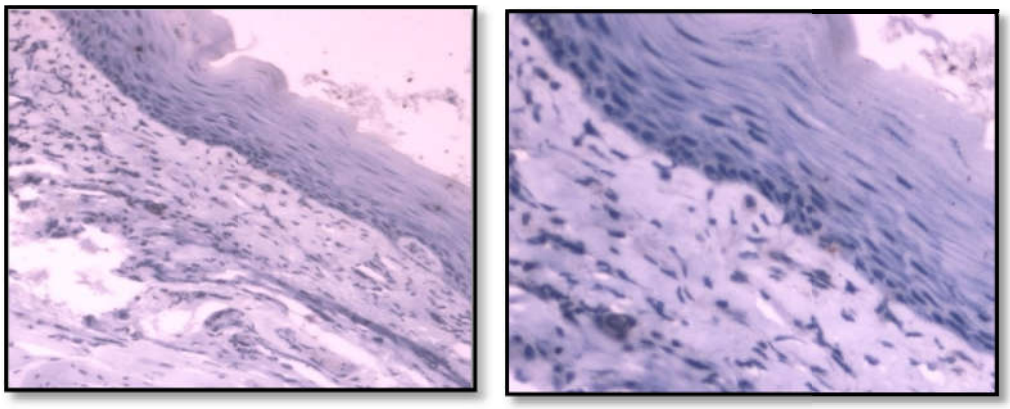


Fig 18C and 18D: Survivin expression is absent in normal oral epithelium (100X; 200X)

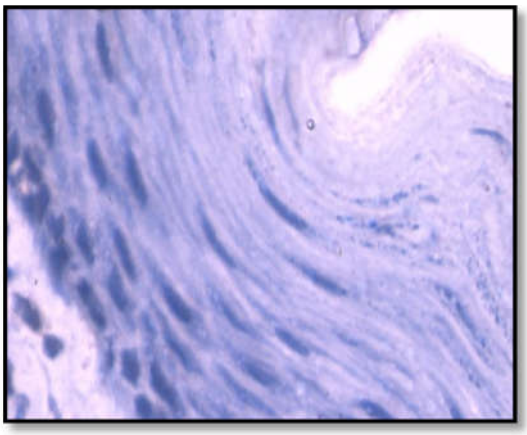


Fig 18E: Survivin expression is absent in normal oral epithelium (400X)

Discussion

OPMD forms a family of clinical or histological alterations of oral epithelium that indicates a risk towards transformation into squamous cell carcinoma. Most oral cancers, though the exact number is unclear, are preceded by a potentially malignant disorder. The estimated global prevalence of OPMD varies from 1% to 5% with an average prevalence rate of 2.6% with regional and geographical variations.[55] Nevertheless, the prevalence rate was reported to be higher in Indian population ranging from 9% to 14.6%.[56] Microscopic examination to identify the presence of epithelial dysplasia in a biopsy sample is currently the reference standard in the assessment of OPMD to assess the risk of developing oral squamous cell carcinoma. Dysplasia, meaning abnormal growth, is the term given to the histopathological changes associated with an increased risk of malignant transformation. Oral mucosal dysplastic changes are characterized by disturbance in epithelial stratification and maturation (architectural features) accompanied by cellular atypia (cytological features).

OL, OSMF and OLP are suggested to have a premalignant potential with varying consistencies. Hence, it becomes imperative to analyze the premalignant features and malignant transformation potential of these lesions for early diagnosis and accurate treatment planning. The ability of a clinician to diagnose a lesion early is further enhanced by histopathology and molecular analytical techniques. A study by Warnakulasuriya et al stated that, despite the availability of many molecular markers for the diagnosis of OPMD, an accurate predictive assessment of the clinical behavior of OPMDs will depend on development of newer markers.[57]

Carcinogenesis is a multistep process that predominantly involves an imbalance between the activation of oncogenes and inactivation of tumour suppressor genes.

Most tumours are the outcome of imbalance of regulatory mechanisms controlling cell cycle progression, cell death/viability balance, and apoptosis. Apoptosis has become an essential tool in cancer research and in establishing new cancer strategies.[29]

Physiologic regulation of apoptosis plays a significant role in embryonic development, tissue morphogenesis, and homeostasis resulting in regulation of cell number and elimination of damaged cells. On the other hand, resistance to apoptotic stimuli is involved in cancer development and progression as well as in autoimmune disorders. Overexpression of IAPs is thought to be a primary mechanism through which tumor cells acquire resistance to apoptosis.

Survivin, an IAP protein, which is of considerable focus in recent times, appears to play a role in maintaining the cancer cell vitality. Any interference in survivin function has resulted in defects in mitotic transition thereby inducing cell death.[58] The role of survivin protein is unique, and have been shown to bind specifically to caspases-3 and -7 leading to inhibition of apoptosis in *in-vitro* system.[59] Furthermore, **Li et al** reported that survivin expresses during the G_2/M phase of the cell cycle and the disruption of survivin-microtubule interactions results in increased caspase-3 activity and accelerated apoptotic cell death.[25] **Ito et al** reported that hepatocellular carcinoma cell lines transfected with survivin show a significant decrease in cells in the Gap_0/G_1 phase and an increase in cells in the synthesis and G_2/M phases.[60] These findings indicate that survivin protein expression may correlate not only with reduced apoptotic cell death but also with an increased proliferative activity of cancer cells. Survivin has been the focus of the scientific community following its selective expression in highly proliferating normal tissues and in cancers of various anatomical

parts of the human body. In OSCC, a high incidence of survivin overexpression has recently been reported.[61] Another interesting finding is that high survivin expression correlates with poor survival rates, thus making it as a better prognostic marker.[29]

In this study, an attempt was made to compare the expression of survivin in OPMD like OL, OSMF and OLP with NOE as control. Several studies have reported an absence of survivin expression in normal oral mucosal tissue specimens.[15, 19, 20, 30, 43, 62] The present study demonstrated negative immunoreactivity in majority of samples, but mild nuclear staining was observed in less than 10% of cells predominantly in the basal layer in 2 samples of NOE. This is in line with few reports where weak nuclear and cytoplasmic expression of survivin was found among sporadic cells of basal and parabasal layers in NOE.[30, 63] The outcome was also consistent with earlier studies which stated that the scanty expression could be due to active mitotic figures. [15, 19, 20, 30, 43, 62]

On the other hand, **Negi at el** has reported survivin positivity in almost 20 percent of normal tissue specimens and attributed this to higher proliferative capacity of these tissues.[48] Likewise, **Tanaka et al** pointed out that the survivin immunoexpression in normal oral epithelium is due to the presence of active mitotic cells.[31] It was demonstrated that survivin was present in normal hematopoietic cells, immune cells, vascular endothelial cells, polymorphonuclear neutrophils, T lymphocytes, melanocytes, keratinocytes, neurons, and other cells of the mammalian brain.[38] In contradiction to our findings, a study by **Chaiyarit et al** demonstrated significant expression of survivin in NOE and attributed to processing errors in fixation, antigen retrieval techniques, types of primary antibodies and detecting systems.[45] Similar

observation was also reported by **Lodi et al** in which all samples of NOE showed varied survivin mRNA expression, typically lower level of expression.[46]

The present study demonstrates survivin immunoreactivity in most cases of OL (87%) with 2 samples (one case each of mild and moderate dysplasia) showing negative IRS. This was comparatively higher, as earlier reports had survivin immunopositivity in the range of 33% to 65% for OL.[13, 31, 32, 48] Some studies have demonstrated complete expression of survivin in epithelial dysplasia.[20, 39, 44] The immunoreactivity varied from mild to moderate with a single case of histopathologically confirmed CIS demonstrating stronger expression. Majority of OL cases selected in this study were mildly dysplastic, while 3 of them had moderate dysplastic features. **Gayathri et al** demonstrated a progressive increase in survivin expression based on predominant staining pattern and histological grading.[51] Although our study demonstrated stronger expression of survivin in one case of CIS, a correlation between the histological grading and survivin expression couldn't be established based on the parameters measured in mild and moderate dysplasia, considering the small sample size utilized in this study.

Survivin seems to exist in two subcellular pools, cytoplasmic and nuclear. Majority of OL samples in our study demonstrated nuclear expression of survivin (73.3%). Conversely, most studies had reported cytoplasmic expression of survivin in oral pre-malignant lesions, especially oral leukoplakia.[13, 19, 20, 29, 30, 31, 39] There are two different opinions regarding the significance of survivin nuclear positivity. Despite the fact that nuclear expression of survivin is an unfavorable factor for prognosis in various tumors and OPMDs occurring in humans, some authors have proposed survivin nuclear positivity as a favorable prognostic marker.[19] However, the authors

demonstrated observer variance in analysing survivin expression in nucleus and cytoplasm and suggested varied analytical procedures like western blots and dilution of antibodies to establish any unusual observation. **Stauber et al** stated that survivin can traverse between the nucleus and cytoplasm and may play a cytoprotective role while in the cytoplasm and likewise play a role in cell division while in the nucleus. This bifunctional role of survivin is dependent on various factors like nuclear export receptors, chromosomal passenger complexes and basic amino acids.[64] **Grabowski et al** demonstrated that the survivin was mainly localized in the cytoplasm of basal cells in normal squamous epithelium but nuclear survivin expression was found in high grade dysplasia of the esophageal tissue.[65] While our study demonstrates increased expression of nuclear survivin, and minimal cytoplasmic expression, the prognostic significance of nuclear and cytoplasmic expression cannot be established with a small sample size. It is of utmost importance that a large scale study to analyse the prognostic outcome of nuclear or cytoplasmic survivin is advised in future to clear the ambiguous situation persisting at present. This could be considered as a shortcoming of our study due to the small sample size.

Survivin was predominantly distributed in the basal and parabasal layers of OL. Nevertheless, it was localised in the parakeratin/keratin layers and the prickle cell layers in most studies.[13, 19, 31, 32] **Grabowski et al** demonstrated that the limitation of survivin in the basal layers of esophageal epithelium is due to the role played by survivin in cellular proliferation.[65]

Almost 46.7% of OL samples had survivin immunopositivity in less than 10% of cells. In 46.7% samples, survivin was expressed in 10-29% cells while a single sample showed positivity in 30-59% cells. No significant difference was noted in the

immunopositivity patterns between our study and earlier studies.[13, 20, 30, 32, 36] While survivin immunopositivity cannot be considered as a valid end point to measure the expression considering the subjective bias, it was used as an indicator in earlier studies. [13, 20, 30, 32, 36]

This study demonstrated strong staining intensity in most samples (46.7%) of OL followed by moderate (33.3%) and mild (20%) staining intensity. Whereas the study by **Gayathri et al** demonstrated predominantly moderate (37%) staining intensity followed by mild (33%) and strong (17%) staining intensity in OL.[51] While the moderate staining is consistent with this study, the staining intensity in other categories did not provide a consistent result. This could be attributed to the fact that the measure of staining intensity could not be considered as an endpoint measurement of survivin expression due to the significant variations present during sample collection, fixation, processing, staining and observation.

Oluwadara et al demonstrated high expression levels of survivin in epithelial dysplastic lesions in comparison to OLP while our study demonstrated almost similar immunoreactivity for survivin in both OL and OLP.[44]

A high potential for malignant transformation was demonstrated in OSMF cases by **Murti et al**[66] and **Ekanayaka et al**.[67] **Murti et al** stated that the malignant transformation rate rose from 4.5% to 7.6% with a 2-year increase in observation period.[66] **Zhou et al (2008)** demonstrated 30% expression in early OSMF, 46.7% in moderate OSMF, and 66.7% in advanced stage of OSMF.[42] **Zhou et al**, in another study in 2010, demonstrated 20% expression in early stages, 45% in moderate OSMF, and 65% in advanced stages of OSMF.[15] While the percentage expression was progressively increasing as the OSMF progressed, the degree of survivin expression

(mean survivin score) did not alter significantly between different stages as the authors considered that the classification of OSMF into stages was based on the degree of fibrous change in subepithelial connective tissue and not based on the degree of dysplasia.[15, 42]

In OSF and OSCC originated from OSMF tissues, survivin was mainly localized in the nucleus with different brown granules.[15, 42] In our study, survivin location in OSMF cases was predominantly noted to be nuclear and distributed in the basal and parabasal layers in majority of the samples. However, the distribution pattern varied according to the stages of OSMF in previous studies. In the early and moderately advanced stage of OSMF, the survivin staining was weakly expressed in basal and prickle layer, while the advanced OSMF had stronger expression in basal, prickle and granulosal layer cells.[15, 42] The intensity of survivin positivity was in accordance with the observation made by Zhou et al.[15, 42]

The malignant potential of OLP varied from 0.4% to 4.9% in earlier studies.[66, 68, 69, 70] An increased occurrence of OSCC had been reported in cases of erosive and atrophic types of OLP.[71, 72, 73] Earlier studies demonstrated survivin expression in OLP to be 64.3%[44], 97%[45], and 95%[50]. The present study had survivin expression in 100% samples. The majority of cases showed moderate immunoreactivity in the basal and parabasal layers of epithelium while the study by **Suganya et al** demonstrated mild to moderate survivin expression predominantly in the basal layer.[50] In the study by **Chaiyarit et al**, OLP demonstrated survivin expression in the nuclear compartment predominantly with one third cases demonstrating both nuclear and cytoplasmic positivity.[45] Likewise, our study also reported survivin positivity predominantly in the

nuclear compartment in 66% samples, both nuclear and cytoplasmic compartments in 20% samples and in cytoplasmic compartment in 14% of samples.

The survivin staining intensity was moderate in 60% samples, strong in 27% samples, and mild in 13% of samples. No significant deviation has been noted in comparison with the previous studies.[44, 45, 50]

Intergroup comparison of survivin expression between OL, OSMF and OLP was not performed in any of the earlier studies. Few studies compared the expression of survivin between two OPMD or between OPMD and OSCC with NOE as a control. Studies with intergroup analyses performed earlier includes **Oluwadara et al** (OL, OLP, OSCC and NOE),[44] **Zhou et al** (OPMD, OSCC, and NOE),[15] **Zhou et al** (OPMD, OSCC, and NOE),[42] and **Gayathri et al** (OL, OSCC, and NOE).[50] Significant associations were demonstrated between the pathology under investigation against the NOE and OSCC in these studies.

Intergroup comparison using IRS in our study revealed significant difference between OL and OLP and between OSMF and OLP. However, no significant difference was observed between OL and OSMF. While most of the OL lesions were cases of mild dysplasia, majority of the OLP cases were erosive OLP. It has been documented that the malignant potential amongst the OLP lesions is comparatively high in erosive OLP.[69] The higher IRS scores in OLP group can be attributed to the fact that the lesions that were selected for IHC were predominantly erosive OLP and they indeed have a higher malignant potential than the cases of OL which were predominantly classified as mild dysplasia based on histopathology.

The distribution of survivin expression in different epithelial layers was insignificant between OL and OSMF and between OSMF and OLP but there was a significance of difference between OL and OLP. While the expression of survivin in majority of OL cases was noted to be in the basal and parabasal layers, almost 33% of OL cases showed expression restricted only to the basal layer of the epithelium. In contrast, all the cases of OLP showed expression in the basal and parabasal layers. There was one case of histopathologically confirmed CIS which showed expression across the full thickness of the epithelium. This shows that the expression of survivin indeed correlates with the progression of dysplastic features across the epithelium. In cases of OSMF, most of the cases showed expression in the basal and parabasal layers. It has been demonstrated in the earlier studies that about 7-26% occurrence of epithelial dysplasia in cases of OSMF.[10, 66] **Zhou et al** has proposed for further studies related to the molecular mechanism of survivin overexpression in cases of OSMF.[15, 42]

The mean immunopositivity showed significance between OSMF and OLP but was insignificant between the other two lesions. No significance could be evaluated between the groups based on staining intensity. Most cases of OLP (80%) showed positive expression in the 10-29% range while 50% cases of the OSMF group showed expression in less than 10% of the cells. This can be again attributed to the fact that the malignant transformation in OLP and OSMF are significantly different. This attains more importance as most OLP cases included in the study were erosive OLP and the 13% of OLP cases which showed expression in less than 10% of cells were reticular OLP. However, in a study by **Hsue et al**, malignant transformation potential of epithelial dysplasia was at 4.82%, OSMF at 1.9%, and OLP at 2.10%. However, the mean duration of transformation was significantly lower for OLP (14.7 months) as

compared to OSMF (52.3%) and epithelial dysplasia (28.2%).[74] In another study by **Ho et al**, the malignant transformation of OPMDS were found to be 24.2% in epithelial dysplasia while none of the cases of OSMF underwent malignant transformation. Increased immunopositivity can be considered to demonstrate an increase in malignant transformation potential but as the measurement of immunopositivity alone is very subjective, further large scale studies may be required before arriving at a consensus.[75]

In our study, significant difference was noted between OL, OSMF and OLP against the NOE as control. However, this differential expression of survivin in normal versus malignant tissue is a strong advocate for development of survivin-based cancer drugs. Therapeutic modality based on a survivin promoter was used to direct expression of other apoptotic genes selectively in tumor cells and it was demonstrated that using bax mutants in combination with survivin promoter for tumor-targeted suicide gene was effective in vitro.[76]

Few shortcomings can be found in our study of which the significant one is the small sample size followed by convenient sampling of specimens from the available archival blocks. While the histopathological grading of OL, OSMF, and OLP was performed, uniform numbers of samples in each grade were unavailable and hence, an intralesional comparison of different histological grades with equal samples was not performed. However, we have correlated the survivin expression patterns with the available histopathological information. While the inclusion of three different lesions with NOE as a control did provide significant information regarding survivin and its role in malignant transformation, we advocate researchers to conduct a large scale study amongst the OPMDs to further enhance the knowledge regarding survivin

immunoexpression and its diagnostic and prognostic significance in each lesion and between different lesions.

In recent times, various modalities of cancer therapeutics including antisense technology, dominant negative constructs, ribozyme technique and histone deacetylase inhibitors are being tested in various studies. Antisense technology includes usage of antisense oligonucleotides, small interfering RNAs, and short hairpin RNAs are being used to inhibit expression of target genes. Dominant negative mutants for survivin (eg. T34A) are developed based on the rationale where the mutants compete with regular cellular proteins for the target and inhibits the function of the survivin. Ribozyme (ribonucleic acid enzyme) have been developed to cleave the survivin mRNA which results in inhibition of translation and subsequent restriction of tumor growth. Evidence also suggests that histone deacetylases like clamydocin and LAQ824 downregulates survivin levels and induces apoptosis of tumor cells.[77]

Conclusion

CONCLUSION

The oral cavity is a very important location for many reasons. It is easily accessible for inspection and sampling without any major risk to the patient. The need for advanced techniques makes it suitable for studying different cellular mechanisms. OPMDs in the mucosa are easy to detect and clearly visible to the healthcare provider. Thus, OPMDs provide us with an excellent model for studying the malignant transformation process and the development of cancer. It is very important to prevent malignant change in people diagnosed with OPMDs, but the hazard ratios of various OPMDs and the rate of malignant transformation are not well established. First and foremost, a detailed study of OPMDs with a prognostic marker established in various other premalignant disorders and cancers has to be evaluated. The rationale behind this study was to identify the significance of such a biomarker across the most common OPMD lesions based on different parameters.

Comparison of survivin across OL, OSMF, OLP and NOE was done in this study. Survivin was not/minimally expressed in normal oral tissue samples, consistent with many studies which had repeatedly proved that survivin is not expressed in normal tissues that do not show high proliferative activity. Oral mucosa, especially gingiva, does not demonstrate a high proliferative activity except for few mitotic cells which showed positivity for survivin. Survivin was expressed in all the OPMDs including OL, OSMF and OLP. Significant difference was observed in the immunoexpression patterns of OPMD in comparison with NOE.

As discussed earlier, the dysplastic changes and significant number of mitotic figures in the OPMDS resulted in an increased expression of survivin in comparison to the normal mucosal specimens. Significant difference was observed in the immunoreactivity patterns of OLP compared to OL and OSMF. Most OLPs which

were included in the study were of the erosive type while most OLs included in the study were mild dysplasias. This can be attributed to the significant difference of expression between OL and OLP. Considering the OSMF and OLP, OLP has been demonstrated to have a significantly higher malignant transformation potential than OSMF which can be attributed to the increased expression of survivin in OLP. Significant difference was observed in the distribution of survivin immunoexpression of OL compared to OLP. This can be probably due to the fact that majority of the mild dysplasia cases of OL demonstrated survivin expression only in the basal layers in comparison to the OLP cases, all of which demonstrated survivin expression in both the basal and parabasal layers.

Significant difference was also found in the mean immunopositivity of OSMF compared to OLP. This may be due to the fact that most OLP cases included in the study were erosive OLP and the 13% of OLP cases which showed expression in less than 10% of cells were reticular OLP. However, in a significant number of OSMF cases, less than 10% cells showed survivin immunopositivity. OSMF is a disease of the connective tissue and epithelium may not show dysplastic changes until the later stages of the disease.

To summarize, large studies are needed to corroborate the prognostic impact of survivin expression in OPMDs and OSCC, however, this dissertation suggests that survivin detection may provide a new tool to identify OPMDs in patients at high risk of unfavorable outcome. This is particularly relevant for the segregation of survivin expression in OPMD patients with an ambiguous histological outcome, the group for which new molecular indicators of dysplastic changes are needed with priority. On the other hand, development of anti-survivin molecules may provide a novel

therapeutic approach for patients by increasing the sensitivity of cells to apoptosis-inducing stimuli.

To conclude, the significant new knowledge forthcoming from this dissertation needs to be brought into the diagnostic component of oral cancer. The objective of healthcare providers is to reduce the morbidity and eliminate mortality of patients with OPMDs and OSCCs.

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Annexure

ANNEXURE 1

INSTITUTIONAL ETHICAL COMMITTEE

Best Dental Science College and Hospital
Ultra Nagar, Madurai - 625 104.
RECOGNIZED BY DENTAL COUNCIL OF INDIA, NEW DELHI
AFFLIATED TO THE TAMILNADU Dr. M.G.R MEDICAL UNIVERSITY, CHENNAI)

IRB/IEC Reference No: 2016-STU-BrVI-RJV-14

Project title: Expression of survivin in oral potentially malignant disorders: a retrospective immunohistochemical study

Principal Investigator: Dr. Rajanna Venkatraman. M,

PG student

Review: New/Revised/Expedited

Date of Review: 27/09/2016

Date of previous review, if revised application:

Decision of the IEC/IRB:

- Provisional approval to conduct the study is being given
- The results of this study, along with summary are to be submitted for obtaining final approval

Recommended time period: one year (28-09-17)

PRINCIPAL
BEST DENTAL SCIENCE COLLEGE
MADURAI-625104

INSTITUTIONAL REVIEW BOARD SIGN DATE BDSC

ETHICS COMMITTEE SIGN DATE BDSC

Signature of Member Secretary

NB:

- **Inform IRB/IEC immediately in case of any issue(s)/adverse events**
- **Inform IRB/IEC in case of any change of study procedure, site and investigator**
- **This permission is only for the period mentioned above**
- **Annual report to be submitted to IEC/IRB**
- **Members of IEC/IRB have right to monitor the trail with prior intimation**

ANNEXURE 2

Immunoexpression of Survivin in Oral Leukoplakia group (Group 1)

S.No	Category	Site	Age	Gender	Intensity	Immunopositivity %	Stain location	Distribution	IRS
1	OL	Buccal mucosa	45	Male	Strong	30-59%	Entire thickness	Cytoplasmic and nuclear	Severe
2	OL	Buccal mucosa	55	Male	Mild	10-29%	Basal	Nuclear	Mild
3	OL	Buccal mucosa	57	Male	Strong	<10%	Basal	Nuclear	Mild
4	OL	Buccal mucosa	41	Male	Moderate	<10%	Basal	Nuclear	Mild
5	OL	Buccal mucosa	65	Male	Strong	10-29%	Basal and Parabasal	Nuclear	Moderate
6	OL	Buccal mucosa	40	Male	Strong	10-29%	Basal and Parabasal	Nuclear	Moderate
7	OL	Gingiva	52	Male	Strong	10-29%	Basal and Parabasal	Nuclear	Moderate
8	OL	Lip	35	Male	Moderate	10-29%	Basal and Parabasal	Cytoplasmic	Moderate
9	OL	Buccal mucosa	56	Male	Strong	10-29%	Basal and Parabasal	Nuclear	Moderate
10	OL	Buccal mucosa	32	Male	Moderate	<10%	Basal	Nuclear	Mild
11	OL	Buccal mucosa	67	Male	Strong	<10%	Basal and Parabasal	Nuclear	Mild
12	OL	Buccal mucosa	44	Male	Moderate	<10%	Basal and Parabasal	Cytoplasmic	Mild
13	OL	Buccal mucosa	57	Male	Mild	<10%	Basal and Parabasal	Cytoplasmic	Negative
14	OL	Buccal mucosa	39	Male	Moderate	10-29%	Basal and Parabasal	Nuclear	Moderate
15	OL	Gingiva	43	Male	Mild	<10%	Basal	Nuclear	Negative

ANNEXURE 2

Immunoexpression of Survivin in Oral Submucous Fibrosis group (Group 2)

S.No	Category	Site	Age	Gender	Intensity	Immunopositivity %	Stain location	Distribution	IRS
16	OSMF	Buccal mucosa	65	Female	Strong	10-29%	Basal and Parabasal	Cytoplasmic and nuclear	Moderate
17	OSMF	Buccal mucosa	40	Male	Mild	<10%	Basal and Parabasal	Nuclear	Negative
18	OSMF	Buccal mucosa	60	Female	Moderate	10-29%	Basal and Parabasal	Nuclear	Moderate
19	OSMF	Buccal mucosa	34	Male	Mild	<10%	Basal and Parabasal	Nuclear	Negative
20	OSMF	Buccal mucosa	55	Male	Mild	<10%	Basal and Parabasal	Nuclear	Negative
21	OSMF	Buccal mucosa	57	Female	Moderate	10-29%	Basal and Parabasal	Nuclear	Moderate
22	OSMF	Buccal mucosa	50	Male	Mild	<10%	Basal and Parabasal	Nuclear	Negative
23	OSMF	Buccal mucosa	47	Male	Moderate	10-29%	Basal and Parabasal	Nuclear	Moderate
24	OSMF	Buccal mucosa	40	Female	Moderate	<10%	Basal and Parabasal	Nuclear	Mild
25	OSMF	Buccal mucosa	45	Male	Mild	<10%	Basal	Nuclear	Negative
26	OSMF	Buccal mucosa	27	Male	Moderate	<10%	Basal and Parabasal	Nuclear	Mild
27	OSMF	Buccal mucosa	65	Female	Strong	10-29%	Basal and Parabasal	Cytoplasmic and nuclear	Moderate
28	OSMF	Buccal mucosa	51	Female	Moderate	10-29%	Basal and Parabasal	Nuclear	Moderate
29	OSMF	Buccal mucosa	31	Male	Mild	10-29%	Basal and Parabasal	Nuclear	Mild
30	OSMF	Buccal mucosa	59	Female	Mild	<10%	Basal	Nuclear	Negative

ANNEXURE 2

Immunoexpression of Survivin in Oral Lichen Planus group (Group 3)

S.No	Category	Site	Age	Gender	Intensity	Immunopositivity %	Stain location	Distribution	IRS
31	OLP	Buccal mucosa	40	Female	Strong	10-29%	Basal and Parabasal	C + N	Moderate
32	OLP	Buccal mucosa	27	Female	Strong	30-59%	Basal and Parabasal	C + N	Severe
33	OLP	Buccal mucosa	56	Male	Strong	10-29%	Basal and Parabasal	C + N	Moderate
34	OLP	Buccal mucosa	34	Female	Moderate	10-29%	Basal and Parabasal	Nuclear	Moderate
35	OLP	Buccal mucosa	37	Female	Mild	<10%	Basal and Parabasal	Nuclear	Negative
36	OLP	Tongue	48	Female	Moderate	10-29%	Basal and Parabasal	Nuclear	Moderate
37	OLP	Buccal mucosa	45	Female	Moderate	10-29%	Basal and Parabasal	Cytoplasmic	Moderate
38	OLP	Buccal mucosa	24	Male	Moderate	10-29%	Basal and Parabasal	Nuclear	Moderate
39	OLP	Buccal mucosa	44	Male	Moderate	10-29%	Basal and Parabasal	Nuclear	Moderate
40	OLP	Buccal mucosa	35	Female	Moderate	10-29%	Basal and Parabasal	Cytoplasmic	Moderate
41	OLP	Buccal mucosa	51	Male	Mild	<10%	Basal and Parabasal	Nuclear	Negative
42	OLP	Buccal mucosa	38	Female	Strong	10-29%	Basal and Parabasal	Nuclear	Moderate
43	OLP	Buccal mucosa	47	Female	Moderate	10-29%	Basal and Parabasal	Nuclear	Moderate
44	OLP	Buccal mucosa	29	Male	Moderate	10-29%	Basal and Parabasal	Nuclear	Moderate
45	OLP	Buccal mucosa	43	Male	Moderate	10-29%	Basal and Parabasal	Nuclear	Moderate

ANNEXURE 2

Immunoexpression of Survivin in Normal Oral Epithelium group (Group 4 - Control)

S.No	Category	Site	Age	Gender	Intensity	Immunopositivity %	Stain location	Distribution	IRS
46	NOE	Buccal mucosa	27	Female	Mild	<10%	Basal	Nuclear	Negative
47	NOE	Gingiva	45	Male	Nil	Nil	Nil	Nil	Negative
48	NOE	Gingiva	25	Male	Nil	Nil	Nil	Nil	Negative
49	NOE	Buccal mucosa	38	Female	Nil	Nil	Nil	Nil	Negative
50	NOE	Gingiva	42	Female	Nil	Nil	Nil	Nil	Negative
51	NOE	Buccal mucosa	29	Male	Nil	Nil	Nil	Nil	Negative
52	NOE	Alveolar mucosa	52	Female	Nil	<10%	Basal	Nuclear	Negative
53	NOE	Buccal mucosa	35	Male	Nil	Nil	Nil	Nil	Negative
54	NOE	Buccal mucosa	26	Female	Nil	Nil	Nil	Nil	Negative
55	NOE	Gingiva	23	Male	Nil	Nil	Nil	Nil	Negative
56	NOE	Buccal mucosa	29	Male	Nil	Nil	Nil	Nil	Negative
57	NOE	Gingiva	46	Female	Nil	Nil	Nil	Nil	Negative
58	NOE	Gingiva	32	Male	Nil	Nil	Nil	Nil	Negative
59	NOE	Alveolar mucosa	39	Female	Nil	Nil	Nil	Nil	Negative
60	NOE	Gingiva	41	Female	Nil	Nil	Nil	Nil	Negative